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THE CARDIOVASCULAR LESIONS
OF
COPPER DEFICIENCY

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Index

	<u>Page</u>
Introduction	3
Review	4
General Experimental	11
The production of copper deficiency in swine.	
Experiment 1.	14
The morphological lesions of the cardiovascular system in copper deficiency.	
Experiment 2.	33
The mechanical properties of the intact aorta.	
Experiment 3.	40
The mechanical properties of isolated aortic elastin.	
Experiment 4.	50
The mechanical properties of isolated aortic collagen.	
Experiment 5.	65
The mucopolysaccharide ground substance and the mechanical properties of the aorta.	
Experiment 6.	79
The repair of vascular defects in copper deficient swine treated with copper.	
Discussion	83
Summary	90
Acknowledgements	92
References	94

Introduction

In 1928, as part of a series on iron in nutrition, Hart, Steenbock, Waddell and Elvehjem published their finding that copper would reverse the anaemia which developed in rats fed a diet of whole cows' milk, supplemented with iron. Although copper was a known constituent of both plant and animal life, this was the first reported evidence of a definite function of copper in the animal body. Subsequently Wintrobe and his colleagues in Utah considerably extended the investigation of the role of copper in haematopoiesis in a series entitled "Studies on copper metabolism", which ran to more than 30 articles (Cartwright and Wintrobe, 1964). In one of these, Gubler et al. (1957) commented on the presence of cardiac hypertrophy in copper deficient swine and its possible connection with the sudden heart failure which they occasionally encountered during the experiments. Possibly because the majority of animals in the Utah series were sacrificed, the sudden death of some of them was not closely scrutinised. In 1961, however, Shields, Carnes, Cartwright and Wintrobe drew attention to the frequency of haemopericardium in pigs dying suddenly in copper deficiency, and from this observation, the present study developed.

The dissertation will retain the order of the several experiments with separate pertinent discussions for each, giving the indications for the next experiment.

Review

It is not my intention to give a report in detail on copper metabolism, which has been well reviewed by several authors, but to give a narrower background, pertinent only to this study.

The discovery of the nutritional significance of copper and its relation to iron in haematopoiesis has been covered by Cunningham (1931) and by Elvehjem (1935). In 1950, a symposium at the McCollum-Pratt Institute of the Johns Hopkins University was devoted entirely to copper metabolism and its animal, plant and soil relationships (McElroy and Glass, 1950). More recent reviews on copper metabolism have been made by Scheinberg and Sternlieb (1960); Scheinberg (1961); and Adelstein and Vallee (1961). Underwood (1962) summarised the nutritional aspects of copper, including the manifestations of copper deficiency in animals, which were also covered by Follis (1958).

Copper may exist "in vivo" in several forms, including free ions and combinations with amino acids, purines, pyrimidines, nucleotides, nucleic acids and proteins (Scheinberg and Sternlieb, 1960). It is the last of these, the copper-proteins, which have been most extensively studied and which bear relevance to this work. They include several which have so far defied elucidation of biological function - cerebro-cuprein I of human brain, erythro-cuprein of human red blood cells, haemocuprein of ox and human red blood cells and serum, and a copper protein of liver (Scheinberg, 1961). It is quite possible that they have functions like the other known copper proteins, which behave as oxidative catalysts. These proteins are caeruloplasmin, tyrosinase, the diamine oxidases,

and cytochrome c oxidase.

Coeruloplasmin, one of the serum proteins, catalyzes the oxidation "in vitro" of a series of substances, including ascorbic acid, adrenaline, nor-adrenaline and serotonin (Laurell, 1960). It may also act as an ascorbic acid oxidase "in vivo" (Osaki et al., 1964).

Tyrosinase has oxidative activity toward poly- and monophenols, which include DOPA and tyrosine itself (Scheinberg and Sternlieb, 1960). Its physiological role, particularly in the formation of melanin, has been made clearer by the study of albinos, who possess no demonstrable tyrosinase activity (Harris, 1959).

The diamine oxidases have been isolated from a variety of tissues and plasma and are thought to detoxify certain diamines, formed by bacterial action in the gut, e.g. cadaverine and agmatine, as well as to inactivate physiological substrates such as histamine (Zeller, 1951). Their possible role in the oxidative deamination of lysine is particularly germane to this thesis, and will be discussed in more detail later.

Cytochrome c oxidase is believed to serve as the terminal oxygen "activating" enzyme in cellular respiration. It may differ from other copper oxidases, however, in the uncertainty of the essential role of copper in its activity. It has been suggested that the lowering of cytochrome oxidase activity in copper deficiency is the result of a low level of haem a, which may require copper either for its synthesis or its incorporation into the holoenzyme (Lemberg et al., 1962).

The assessment of the copper status of an animal is made during life by analysis of the blood, and subsequently by examination of

various tissues. The blood copper is divided approximately equally between red cells and plasma. Lahey et al. (1952) give values in swine of $110 \pm 42.0 \mu\text{g} \%$ of copper in red cells and $186 \pm 15.4 \mu\text{g} \%$ in plasma. The copper in red blood cells is part of such proteins as haemo- and erythrocyte, and cytochrome oxidase, and is maintained at a remarkably constant level (Gubler et al., 1953). The plasma copper is predominantly in the form of caeruloplasmin, and the remainder as a loosely bound, labile albumen complex, which accounts for 4, 1, 12 and 42 per cent of plasma copper in man, rat, dog and swine respectively (Gubler et al., 1953). The main storage organ for copper is the liver, especially in the new-born, with lesser storage in brain, heart and kidney, in several animal species (Cunningham, 1951).

A deficiency of body copper is associated with lesions in several different organ systems, and their extent depends on factors such as species, age, environment and duration (Underwood, 1962). It has been suggested that this may reflect the relative extent to which each copper enzyme system fails, when the available copper is no longer sufficient for all of them (Marston, 1950). The association of each syndrome with a particular enzyme deficiency is still largely speculative, but the occurrence of a lesion of the cardiovascular system in copper deficiency seems to fit well with this possibility. The previously recognized disorders are not being emphasised in this work and so will only be mentioned briefly.

1. Copper and the blood

The anaemia of copper deficiency was first demonstrated in the rat (Hart et al., 1928) and has been described subsequently in every

species in which copper deficiency has been observed. Copper is an essential component of red blood cells, in the form of haemo- or erythrocuprein (Mann and Keilin, 1938; Markowitz et al., 1959), and its deficiency also shortens erythrocyte survival time (Bush et al., 1956). There is no evidence that the anaemia is produced by failure of synthesis of the haem a prosthetic group of cytochrome oxidase.

2. Copper and the Central Nervous System

An enzootic ataxia of unborn or unweaned lambs in Western Australia was shown by Bennetts and Chapman (1937) to be associated with subnormal levels of copper in the pastures and in the blood and tissues of affected lambs and their dams. It could be prevented by copper supplementation of the pregnant ewe and controlled by top-dressing of pastures with copper compounds (Bennetts and Beck, 1942). A similar condition has been described in several other countries, including Great Britain, where it is known as swayback. This condition has been extensively investigated by Barlow et al. (1960), who concluded that it resulted from an inhibition in the development of nerve cells, myelin sheaths and glia in the brain-stem and spinal cord, and referred to the process as a neuro-dysgenesis. Levels of cytochrome oxidase in the brains and livers of lambs affected with swayback were shown to be significantly reduced by Howell and Davison (1959). This was confirmed by Barlow (1963) who also demonstrated that the most severe reductions coincided with those groups of nerve cells which showed the morphological lesions of the disease. Whilst cytochrome oxidase is presumably required for the majority of neuronal and glial activities, including the formation

of myelin, it would be a gross over-simplification to relate the three - copper deficiency, low cytochrome oxidase and neurodysgenesis - in direct line. In fact the possibility of a primary toxic factor, present in the herbage of some areas, the action of which is greatly increased by a state of copper deficiency, still remains (Underwood, 1962).

3. Copper and the Integumentary System

Achromotrichia has been observed in the copper deficient rat, rabbit, cat, dog, goat, sheep and cow (Underwood, 1962), but not in the pig (Lahey et al., 1952). Although the precise biochemical mechanism has not been worked out, it is possible that this lesion results from failure in the oxidative pathway of tyrosine to melanin, through lack of tyrosinase.

Apart from pigmentation, marked changes in the growth and physical appearance of hair, fur or wool have been noted in copper-deficient rats, rabbits, dogs, cattle and sheep (Underwood, 1962). A feature of this derangement of keratinization in wool, particularly that of Merino sheep in Australia, is loss of natural crimp and mechanical properties (Marston, 1946). Uncrimped or "stringy" wool has more sulphydryl and fewer disulphide groups than normal. The rapidity with which thiol groups are lost and disulphide groups gained after dosing a deficient animal with copper, leaves little doubt concerning the association of copper with the oxidative closure of thiol residues to disulphide linkages, although the specific enzymes involved are not yet known (Marston, 1950).

4. Copper and the Connective Tissues

a. Bone formation

An abnormally fragile skeleton, which resulted from

osteoporosis of varying severity, has been reported in connection with enzootic ataxia of lambs in Australia (Bennetts, 1935), and in copper deficient cattle in New Zealand (Cunningham, 1950), but was not emphasised in swayback lambs in Scotland by Barlow et al. (1960). Experimentally, skeletal changes have been observed in swine (Lahey et al., 1952), dogs (Baxter and Van Wyk, 1953) and chickens (Gallagher, 1957). The lesions in each of these species correspond with those described in detail by Follis (1958). There is osteoporosis associated with an epiphyseal lesion, virtually identical with that seen in scurvy. This is of great interest because the activity of an ascorbic acid oxidase in animals has been ascribed to a copper-protein, caeruloplasmin (vide supra). However no biochemical pathway has yet been worked out in this connection.

b. The Cardiovascular System

A condition known as "falling disease", characterized by sudden death, has been described in cattle in south-western Australia. It has a constant association with copper-poor pastures, and can be prevented by giving copper to affected animals or by treating the herbage with copper (Underwood, 1962). In an experimental herd of cattle reared on such pastures, Bennetts et al. (1948) described the following lesions. The myocardium was pale, soft and flabby. There was no microscopic evidence of degeneration or necrosis of heart muscle fibres, but groups of fibres were atrophic and associated with replacement fibrosis. The severity of the heart lesion increased with the age of the animal. The oldest was killed at 45 months of age and exhibited much greater cardiac fibrosis than the single heifer which died of "falling disease" aged 28 months. The disease has never been reported in

sheep or horses grazing under the same conditions, and sudden deaths in cattle in copper-deficient areas elsewhere have been scarce (Underwood, 1962). In experimentally induced copper deficiency in swine, Gubler et al. (1957) reported the presence of cardiac hypertrophy rather than atrophy. This certainly reflected in large part the hypervolaemic heart failure of a severe anaemia, although the authors maintained that the cardiac hypertrophy exceeded in severity that associated with an equivalent anaemia in iron-deficient pigs. The presence of a severe anaemia was not emphasised in connection with cattle suffering from "falling disease". The difference in heart lesions between experimental swine and range cattle might also have been a reflection of the relative acuteness of the deficiency in swine, which, in the quoted experiment, were all killed before 14 weeks of age. The only other reference to heart lesions in copper deficiency, prior to the present work, was made by Teague and Carpenter (1951), also in the pig. They record that, out of 27 copper-deficient swine, "one other animal died of a coronary condition on the 53rd day".

Low levels of cytochrome oxidase activity have been reported in the hearts of copper-deficient rats, chickens and pigs (Gallagher, 1957; Gubler et al., 1957), and it is probable that this is true for cattle in the "falling sickness" areas. However, the discrepancy in heart lesions from area to area, and species to species, as already mentioned with respect to the brain lesions of the ataxia of lambs, raises the possibility of another factor, other than copper, in the pathogenesis of "falling sickness" in cattle. It is proposed to offer an explanation, in this thesis, for the "coronary conditions" of copper-deficient swine.

General Experimental

The production of copper deficiency in swine

1. Animals

Pigs of mixed breed were obtained from the breeder as litter mates, up to fourteen in number, at ages of two to seven days. The younger animals became copper-deficient earlier but were considerably more liable to fatal respiratory infections and diarrhoea. These illnesses never seemed to be linked to particular organisms and the exhibition of various antibiotics and chemotherapeutic agents did little to affect the outcome. The optimum age of weaning, for minimal neo-natal morbidity and eventual death from copper deficiency at between 60 and 100 days, was four days.

The animals were housed in galvanized iron pens with galvanized iron screen floors, but feeding troughs were made of stainless steel. During the first ten to fourteen days after reception, the piglets were housed in groups of four to six to a pen and warmed by infra-red lamps. Thereafter each pig occupied its own pen.

2. Basal Diet

The basal diet consisted of canned evaporated milk (Carnation Company), diluted 1:1 with water. Deionized distilled water was used as diluent for the first 10 days after receipt of the animals, and thereafter sulphide treated tap water. The tap water, before being added to the milk, was allowed to stand for at least 24 hours in a 5-gallon plastic jug to which were added 10 ml of a 0.36 per cent solution of sodium sulphide. The diet was fed in an amount of 230 ml of diluted milk (167 kg cal) per kg of body weight per animal per day. Half was fed in the morning and half in the late afternoon.

3. Dietary Supplements

(i) Iron

Iron was obtained as carbonyl iron powder, grade RX, from Antara Products, New York. This reduced iron is spectroscopically free from copper. After dissolving in concentrated HCl, deionized water was used to make a solution containing 72 mg of iron per ml. This was fed once a day in an amount of 0.5 ml (equivalent to 36 mg of iron) per kg of body weight.

(ii) Copper

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was dissolved in deionized distilled water in a concentration of 2.0 g per litre. It was fed at a rate of 1 ml (0.5 mg of copper) per kg of body weight per day.

(iii) Other minerals

Seven reagent grade salts were dissolved in deionized distilled water in a total volume of 1 litre. The weight of each salt is given in gm per litre: manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), 1.8; aluminium sulphate ($\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$), 0.6; sodium fluoride (NaF), 3.1; potassium iodide (KI), 3.1; zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 1.8; cobalt nitrate ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), 1.8; nickel acetate ($\text{Ni}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$), 2.8. This supplement was added to the milk in the amount of 0.2 ml per kg body weight per day.

(iv) Vitamins

The following water soluble vitamins were given in mg per kg body weight per day: thiamine hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.2; pyridoxine hydrochloride, 0.2; calcium pantothenate, 0.5; inositol, 0.2; para-aminobenzoic acid, 0.1; biotin, 0.1; pteroylglutamic acid, 0.1; cobalamine, 0.01;

choline chloride, 10.0; and ascorbic acid, 15.0. Three thousand units of vitamin A, 600 units of vitamin D, 1 mg of vitamin E, and 1 mg of vitamin K per kg body weight were given once a week.

The addition of each supplement to the basal diet was varied, and will be specified in the description of each experiment.

Experiment 1

The morphological lesions of the cardiovascular system in copper deficiency

Experimental

Fifty-eight swine of Yorkshire, Duroc Jersey and Chester White cross-breeds, from 8 different litters, were weaned at 2 to 7 days of age and divided into the following groups.

- I (Control) Seventeen animals were fed the basal diet supplemented with iron and copper.
- II (Copper-deficient) Twenty-six animals received the basal diet with iron, but no copper. In order to control a possible effect of total dietary intake on the incidence of lesions, paired feeding was undertaken with 3 pairs of litter-mate piglets, matched as to initial weight. The diet of one member of each pair contained no copper. The other member received copper and therefore fell into Group I (control). Each day the volume of diet consumed by the copper-deficient member was measured, and the following day the control member received the equivalent amount.
- III (Copper-deficient, supplemented) Seven pigs received the basal diet with iron and without copper, but in order to assess possible effects of a lack of other factors, they were further supplemented with additional minerals and vitamins.
- IV (Iron-deficient) To determine if the development of lesions in the copper-deficient group could have resulted from tissue anoxia secondary to severe anaemia, anaemia of like severity

was produced in 8 pigs by feeding the basal diet and copper, but no iron. Periodic phlebotomies, at which 100 to 400 ml of blood were removed, were undertaken in order to accentuate the anaemia.

All the animals were weighed weekly, at which times blood samples were removed by jugular venepuncture. Haematocrit evaluations were made on all blood samples and less frequently the serum copper by the method of Gubler et al. (1952) and the serum iron by the method of Hamilton et al. (1950) were assessed. Periodically the level of copper in the diet was checked by the method of Markowitz et al. (1961). Those animals which did not die spontaneously were killed by exsanguination under sodium pentobarbital anaesthesia. Necropsies were performed immediately or as soon as spontaneous death was discovered. A wide range of tissues, with special emphasis on blood vessels of all dimensions, were fixed in Helly's fluid and in 10% formalin. Following paraffin embedding, sections were stained routinely with haematoxylin and eosin and by the Verhoeff-Van Gieson method for elastin. Selected sections were also stained by the Weigert's elastin, orcein, Masson trichrome, toluidine blue and periodic acid - Schiff methods.

Results

I Control group

All the animals in this group developed normally (Fig. 1) and were killed at the ages noted in Table 1. The serum copper was measured in each one at that time, and the serum iron in 3 of them (Table 1). Both sets of values fell within reported limits for control animals (Lahey et al., 1952). No animal had gross cardiovascular lesions, although one had a heart weight 2.9 S.D. above the normal mean.

Table 1. Data on age, body weight, volume of packed red cells, serum copper and iron values at termination of the experiment

Group	Description	No. Pigs	Age at termination (days)	Body weight	V.P.R.C.* (ml/100 ml)	Serum iron	
						Serum copper µg/100 ml	No. pigs µg/100 ml
I	Control	17	109 ± 22.2 [‡] (70 - 143)	21 ± 6.7 (11.4 - 32.4)	42 ± 8.0 (36 - 50)	172 ± 27.5 (119 - 206)	3 274 (249 - 307)
II	Copper deficient	26	98 ± 22.5 (55 - 127)	16 ± 5.3 (8.8 - 28.2)	24 ± 9.6 (10 - 42) [‡]	13 ± 12.6 (1 - 44)	3 41 (37 - 46)
III	Copper-deficient, supplemented	7	90 ± 13.3 (68 - 99)	15 ± 3.5 (9.6 - 23.0)	15 ± 3.9 (9 - 27)	10 ± 7.6 (1 - 22)	
IV	Iron-deficient	8	91 ± 14.6 (62 - 107)	19 ± 3.6 (10.6 - 26.8)	10 ± 4.0 (6 - 20)	190 ± 18.3 (180 - 208)	8 40 ± 15.4 (24 - 69)

* V.P.R.C. refers to volume of packed red cells.

‡ The mean values ± one standard deviation are given; the values in parentheses refer to range.

† The high value is from a pig that died at 55 days before deficiency was established.

II Copper-deficient group

These pigs gained weight at similar rates to those of the control group, but developed hypocupraemia, hypoferraemia, and anaemia (Table 1). During the course of their deficiency, they developed the marked skeletal abnormalities previously described (Follis et al., 1955), and large haematomas over external pressure areas (Fig. 2). Twenty-three of the animals died. The other 3 pigs (1558, 1563 and 1569) were killed at ages of 77, 120 and 120 days respectively, in order to examine the cardiovascular system histologically before death occurred as a consequence of the experimental conditions. One pig (1549) died before deficiency became manifest, at 55 days of age. The results of the pair-feeding study are given in Table 2.

The principle necropsy findings are summarized in Table 3. Eighteen animals died at ages of 61 to 127 days with haemopericardium, the result of rupture of the aorta, heart, or the coronary or pulmonary arteries. Two other pigs, dying at 105 and 107 days of age, had clear effusions in their serous cavities and peripheral oedema, presumably from heart failure. One of the killed animals also had a massive hydropericardium in association with a cardiac infarct. Three pigs that died between 76 and 96 days of age had marked cardiac hypertrophy, but no evidence of failure or haemorrhage. An exact cause of death in these three was not evident, although one had an organizing pericarditis. With one exception, every pig in this copper-deficient group had distinctive lesions of the heart, aorta or other vessel. These will be described in detail below.

III Copper-deficient, supplemented group

These pigs behaved exactly like those of Group II, copper-



Fig. 1. Control pig, approximately 8 weeks old.

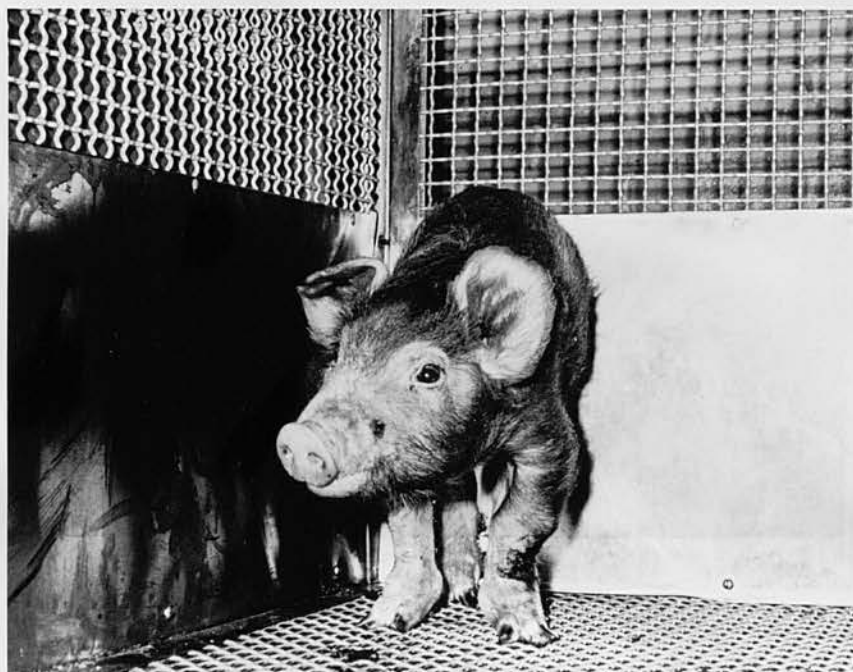


Fig. 2. Copper-deficient pig of same age as Fig. 1., showing bowing of fore-legs. A haematoma over the left carpal joint has ulcerated.

Table 2.
Data on Pair-Fed Animals

<u>Pair</u>	<u>Initial weight (kg)</u>	<u>Final weight (kg)</u>	<u>Final V.P.R.C.* (ml)</u>	<u>Final age (days)</u>
Control	3.2	11.4	37	99
Copper-deficient	3.2	11.6	12	97
Control	2.6	14.4	41	103
Copper-deficient	2.6	13.4	22	102
Control	3.0	15.6	41	100
Copper-deficient	3.0	15.0	10	100

* V.P.R.C. refers to volume of packed red cells.

Table 3.

Incidence of Principal Necropsy Findings

	<u>Group I</u>	<u>Group II</u>	<u>Group III</u>	<u>Group IV</u>
	<u>Control</u>	<u>Copper-</u> <u>deficient</u>	<u>Copper-</u> <u>deficient,</u> <u>supplemented</u>	<u>Iron-</u> <u>deficient</u>
Number of pigs	17	26	7	8
Number of pigs died	0	23	6	4
Gross cardiovascular lesions*	0	25 (96%)†	6 (86%)	0
Haemopericardium	0	18 (69%)	2 (29%)	0
Serous effusions	0	3 (12%)	4 (57%)	1 (12%)

* Exclusive of cardiac hypertrophy (see Table 4).

† Figures in parentheses are percentages of pigs in that group.

deficient, unsupplemented. Six of the seven died spontaneously at ages of 88 to 99 days. Two of these had haemopericardium, the result of rupture of the pulmonary artery in one and of the right atrium in the other. Four had serous effusions and one had pulmonary oedema. The seventh pig was killed at 68 days of age, but failed to show any gross cardiovascular lesions.

IV Iron-deficient group

Four of these animals were maintained until death occurred at from 62 to 102 days. They all developed severe anaemia and hypoferraemia (Table 1), and had markedly hypertrophied hearts. One animal had a 500 ml ascites. The other four were killed at ages 72 to 107 days and also showed cardiac hypertrophy. None of the 8 animals in this group had gross cardiovascular lesions like those of groups II and III.

The Cardiovascular Lesions

Gross examination

The main types of lesion are given in Table 4.

1. Cardiac hypertrophy

The relationship between heart weight and increasing body weight was established in 17 control pigs from Group I, weighing 0.7 to 29.6 kg, and satisfied the equation $H.W. = 0.0391 B.W.^{0.785}$. Within the range of body weights between 10 and 30 kg, the ratio of heart weight to body weight in 15 control pigs was $4.98 (\pm 0.803) \times 10^{-3}$. This ratio was markedly elevated in groups II, III and IV, in all of which anaemia was a pronounced feature, and was directly correlated with the anaemia down to a volume of packed red cells of about 10 ml per 100 ml (Fig. 3). This relationship was

Table 4.
Incidence of Gross Cardiovascular Lesions

	<u>Group I</u>	<u>Group II</u>	<u>Group III</u>	<u>Group IV</u>
	<u>Control</u>	<u>Copper-</u> <u>deficient</u>	<u>Copper-</u> <u>deficient,</u> <u>supplemented</u>	<u>Iron-</u> <u>deficient</u>
Number of pigs	17	26	7	8
Cardiac hypertrophy [*]	0	24 (92%)†	6 (86%)	8 (100%)
Other cardiac lesions	0	11 (42%)	5 (71%)	0
Aortic lesions	0	24 (92%)	6 (86%)	0
Other vascular lesions	0	7 (27%)	3 (43%)	0

^{*} Heart weight greater than 3 S.D. above the normal mean.

† Figures in parentheses are percentages of pigs in that group.

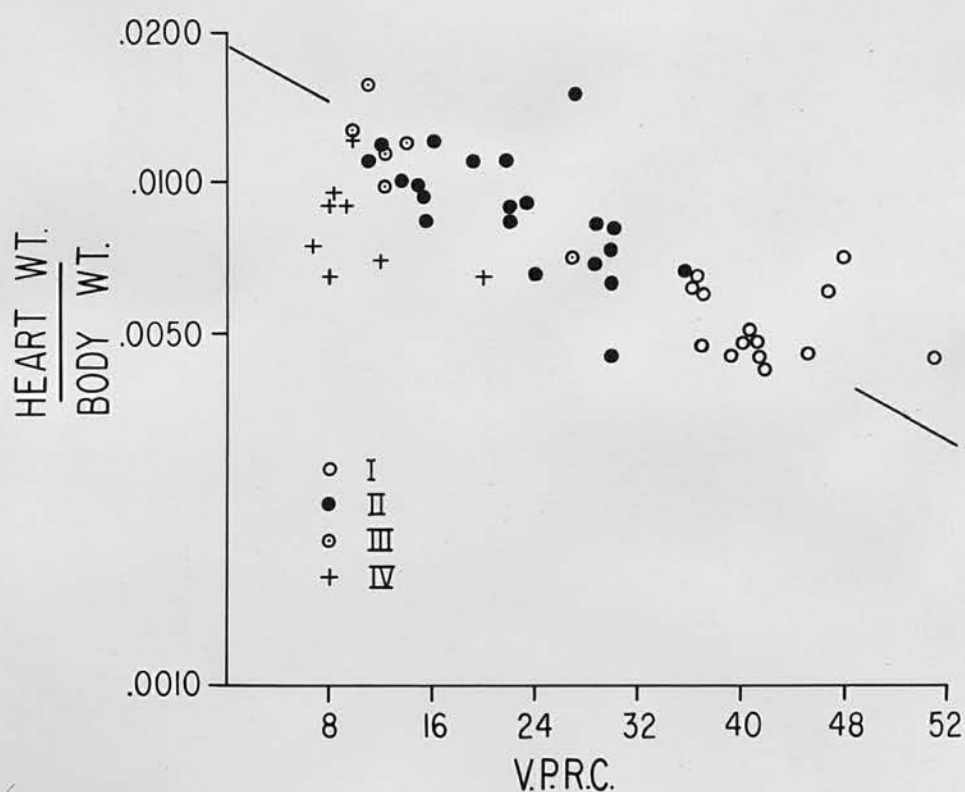


Fig. 3. Relationship of anaemia to relative cardiac weight. Ordinate is the heart weight/body weight on a logarithmic scale. Abcissa is the volume of packed red cells in ml per hundred ml of blood.

irrespective of the type of the deficiency.

2. Other cardiac lesions

a. Left ventricular myocardial infarction was present in three animals, all from Group II (Fig. 4). In one of these the infarct had perforated with massive haemopericardium. In each case there was complete stenosis of a large coronary artery by intramural dissection (see below).

b. Rupture of one or more papillary muscles was present in the left ventricle in 5 pigs, and in the right in 1 (Figs. 5 & 6). This was associated with perforation of apparently normal atrial wall in 2 animals, 1 left-sided, the other right, with massive haemopericardium. In none was there evidence of prior infarction.

c. Large interstitial haemorrhages were found predominantly in the left ventricular myocardium of 4 pigs, and in the sub-epicardial region of four others (Fig. 7). All of these were complicated by haemopericardium.

d. Manual examination of the hearts gave the distinct impression of abnormal myocardial friability. Several attempts were made to measure this by various manometric distension techniques, but without success. Nevertheless other observers repeatedly confirmed this finding, which was also present after prolonged formalin fixation.

3. Aortic Lesions

Rupture of the aorta, present in 9 pigs, occurred invariably in the ascending segment or first part of the arch. Most frequently there was an oblique or transverse split in otherwise

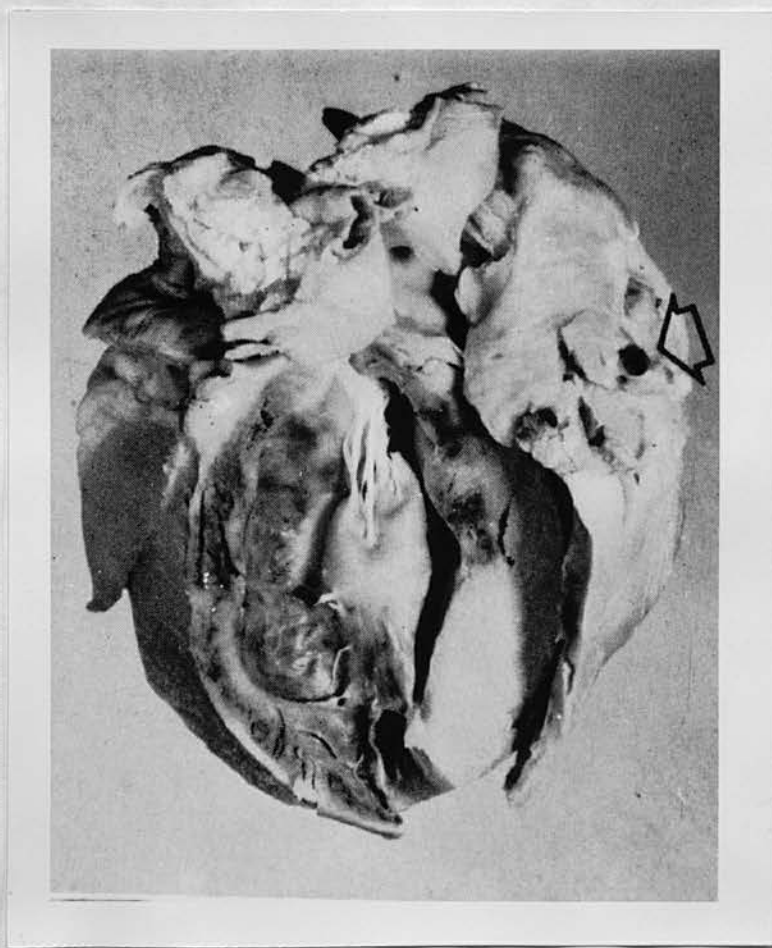


Fig. 4. Myocardial infarct. A coronary artery occluded by dissecting haemorrhage is indicated by arrow.



Fig. 5. Ruptured papillary muscle. The fragment has prolapsed through the mitral valve, as seen from the atrial aspect.

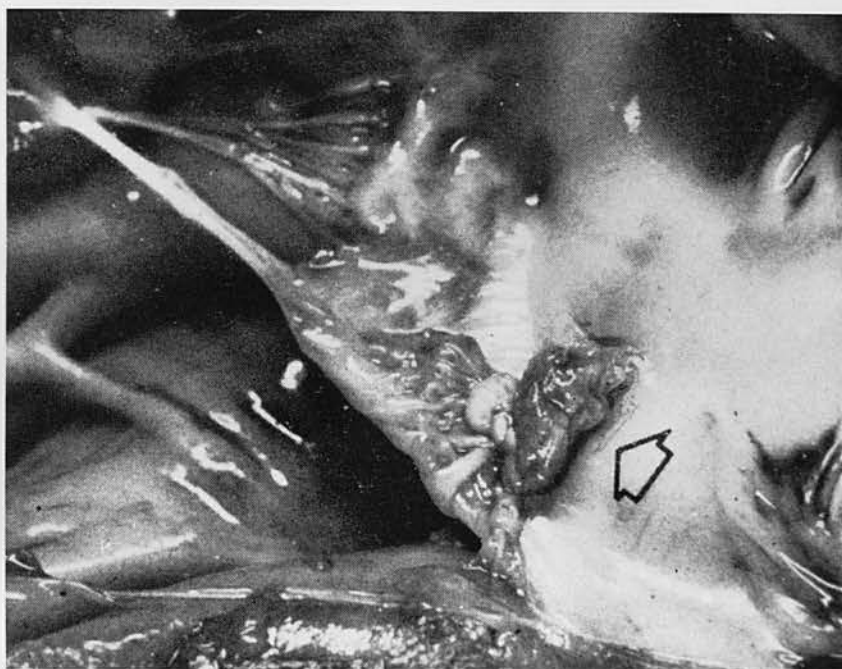


Fig. 6. Ruptured papillary muscle. The ruptured apex of the papillary muscle is shown attached to the reflected posterior mitral leaflet.



Fig. 7. Subepicardial haemorrhage. The haemorrhage, covering the anterior surface of the left ventricle, arose from a ruptured left coronary artery.

normal-appearing intima, extending directly through to the adventitia (Fig. 8). Progressive or retrograde dissection within the media, comparable to that seen in human dissecting aneurysms, was never encountered. In a few animals the aorta was virtually transected. In 9 other animals, similar splits had not completely penetrated the wall.

The descending thoracic aorta was the least seriously affected segment. When present, lesions consisted of small intramural haemorrhages and linear fissuring about the ostia of intercostal arteries (Fig. 9).

The earliest and most regularly occurring lesions were seen in the abdominal aorta. They were present in 91% of the pigs of Groups II and III. In the most florid examples the entire intimal surface was "moth-eaten" by irregular pits and fissures, linear and stellate, frequently haemorrhagic (Figs. 10 & 11). Those obviously penetrated the media to various depths, but never perforated and never dissected beyond a few millimetres. Here too there was a predilection for the ostia of small branches.

In addition to these more obvious lesions, the entire aorta showed a remarkable thickening of the tunica media. This is reflected in the comparative aortic weights shown in Table 5. In both copper-deficient groups the ratio of aortic to body weight is significantly higher than in the control and iron-deficient groups.

In no animal in any of the groups were lesions resembling those of athero-sclerosis noted.

4. Pulmonary artery lesions

Rupture of the main pulmonary artery in 2 pigs resulted from tears exactly like those in the ascending aorta. Five animals in



Fig. 8. Ruptured aorta. The irregular defect is shown in the ascending aorta (arrow). The haemorrhage that surrounds the aorta ruptured into the pericardial sac.

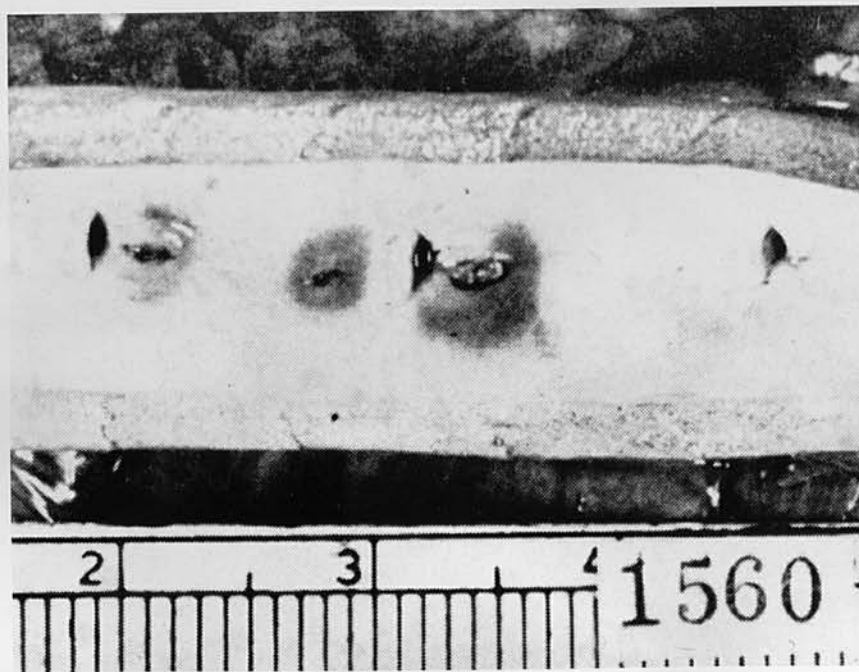


Fig. 9. Intramural haemorrhage in the descending thoracic aorta. The cracks and haemorrhages surround the ostia of intercostal arteries. Note the cut edge of the thickened aortic wall.

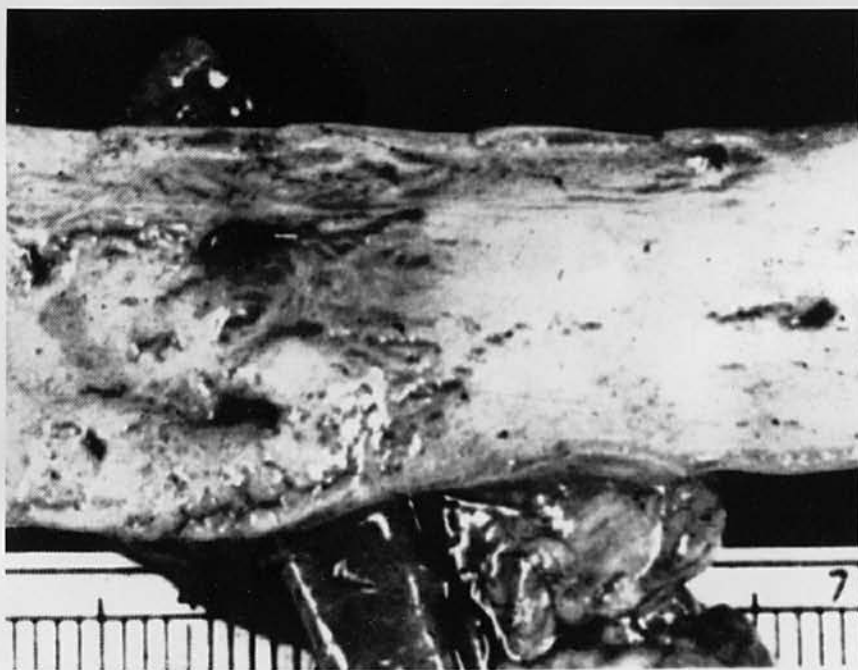


Fig. 10. Fissures in abdominal aorta. Many of the lesions penetrate the media deeply.



Fig. 11. Haemorrhagic pits and fissures in abdominal aorta at bifurcation.

Table 5.
Relative Aortic Weights

<u>Group</u>	<u>No. pigs</u>	<u>Aortic weight/body weight</u> (gm/kg)
I Control	9	$0.803 \pm 0.133^*$
II Copper-deficient	8	1.16 ± 0.032
III Copper-deficient, supplemented	7	1.005 ± 0.079
IV Iron-deficient	7	0.853 ± 0.110

* The mean values \pm one standard deviation are given.

all had haemorrhagic fissures or pitting in the major branches, but in no case did they approximate the severity of those in the corresponding aorta.

5. Other vascular lesions

The coronary arteries were consistently examined and frequently showed small intramural haemorrhages. In 3 pigs, however, these were relatively massive and had resulted in eccentric shifts of the lumen with compression and occlusion, and in each case, associated myocardial infarction (Figs. 4, 12 & 13). Although segments of other muscular arteries were regularly removed for histologic examination, they were not subjected to searching scrutiny, and no obvious gross lesions were noticed.

Microscopic Examination

1. Heart

In those instances in which there were gross myocardial infarcts, the microscopic appearances corresponded. All were recent. Where there was rupture of the myocardium without gross infarction, microscopic changes were surprisingly absent. In particular there was no necrosis, although those cells at the edge of a defect showed acidophilic condensation and loss of striations (Fig. 14). When intramural haemorrhage was the presenting feature, this had occurred around apparently normal fibres.

2. Aorta and pulmonary artery

The lesions in these vessels differed only in degree. The most common defect was an accumulation of amorphous material

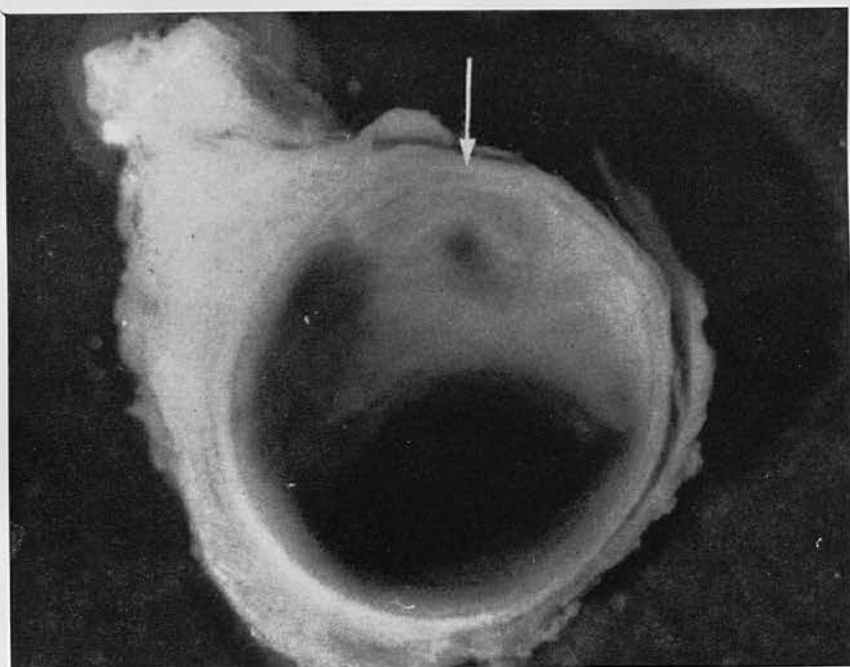


Fig. 12. Coronary artery. A large haemorrhage is contained within the adventitia and has displaced the medial tube to one side (arrow). x 25



Fig. 13. Coronary artery. A dissecting haemorrhage similar to Fig. 12. Section has been made some distance away from the segment of complete occlusion of the lumen. x 30.

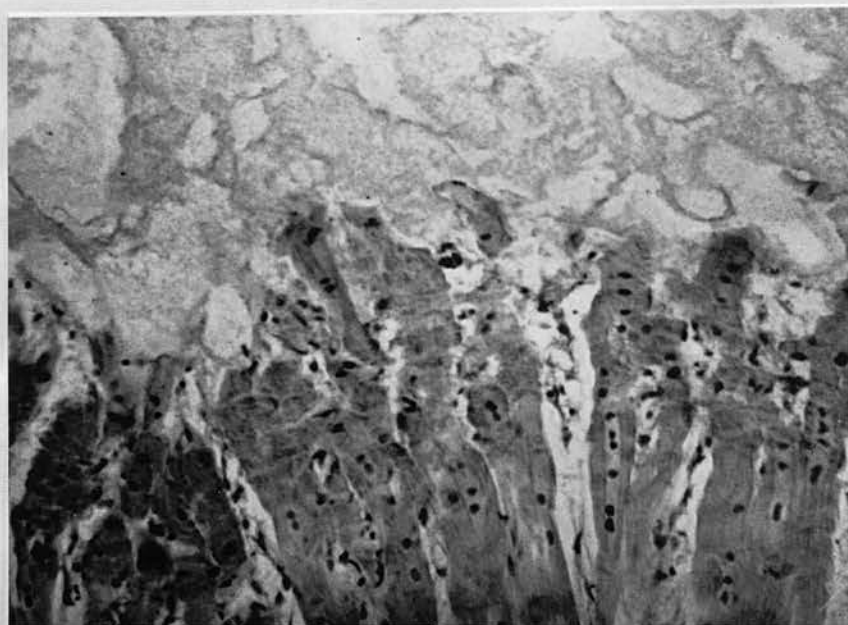


Fig. 14. Ruptured edge of papillary muscle. Histologic section shows ruptured fibres, without evidence of prior necrosis, covered by fresh fibrin. Haematoxylin and eosin stain. x 250.

between the elastic laminae of the media (Fig. 15), and was present in 26 (79%) of the aortas of the copper-deficient groups (I and II). In a few animals the deposition was micro-cystic. The tinctorial characteristics varied from pale and faintly basophilic to a deep eosinophilia. The pale staining material was strongly metachromatic with toluidine blue, whilst the more eosinophilic deposits frequently stained positively with the elastic stains (Fig. 16). Neither material stained with the P.A.S. reaction. Although this accumulation was a most characteristic lesion of the copper-deficient aortas, a similar excess was seen as scattered foci of the pale staining variety in the aortic arches of 2 control (12%) and of 1 iron-deficient pig (12%). Associated with these deposits in 13 (40%) of the aortas from the copper-deficient group, were lesions of the elastic laminae (Fig. 17). These varied from fraying and loss of tinctorial clarity, to rupture and loss of circumferential alignment or complete absence, particularly in the areas of microcyst formation. Such changes were never seen in control pigs, but loss of laminae was present in association with the focal deposition of amorphous material in the iron-deficient pig referred to above.

Dissecting medial haemorrhage was observed in sections of 20 (60%) of the aortas of the copper-deficient groups. This is not a reliable indication that microscopic haemorrhage always preceded rupture, since the sections were often made to illustrate the gross haemorrhages, and the sampling was biased. However, microscopic haemorrhages did occur in association with the elastic defects referred to above in the absence of gross haemorrhage and

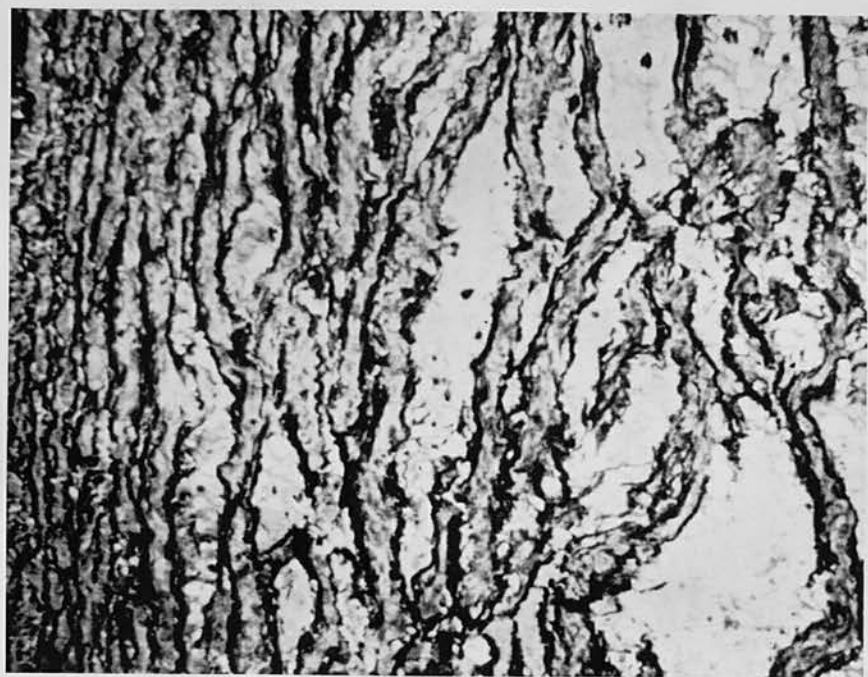


Fig. 15. Thoracic aorta. Histologic section shows separation of elastic laminae by pale-staining amorphous material. Verhoeff elastic tissue stain. x 200.

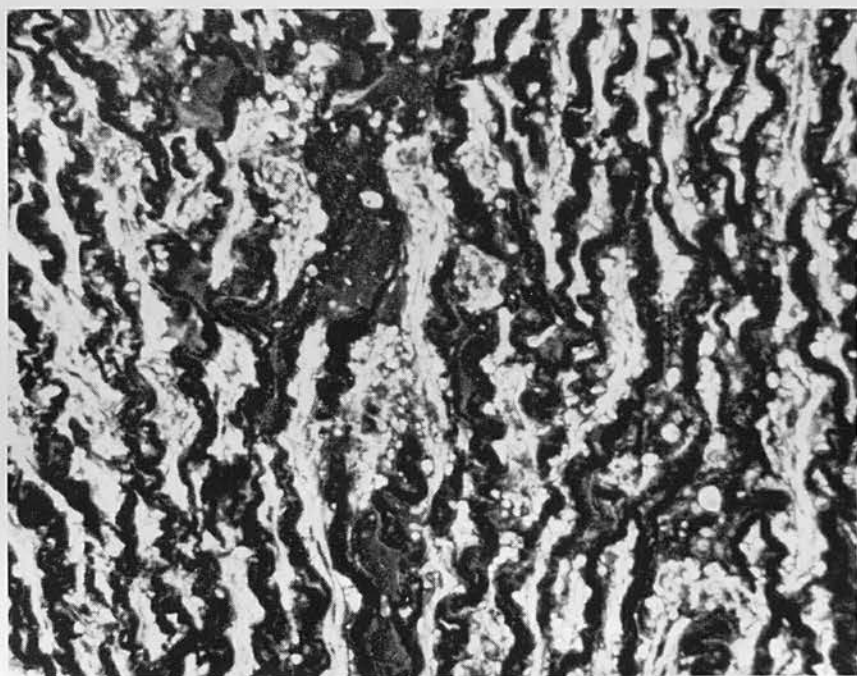


Fig. 16. Thoracic aorta. Interlamellar accumulations of deeply staining amorphous material take the resorcin-fuchsin stain. Weigert elastic and van Gieson stains. x 200.

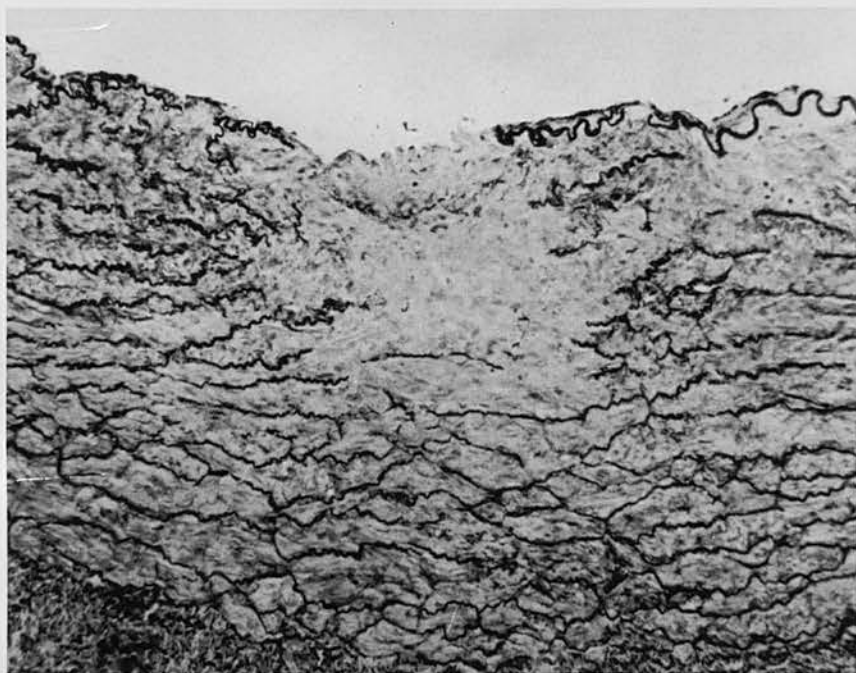


Fig. 17. Abdominal aorta. Ruptured elastic laminae in a healing fissure. Verhoeff elastic tissue stain. x 175.

rupture. None were found in the control or iron-deficient groups.

Small laminar medial necroses were found in the aortas of 7 pigs in Group II. These were invariably on the margins of dissecting haemorrhages and may have been a consequence of the interruption of vasa vasorum by the splits in the media. Collagenous repair of the medial defects was frequently evident, particularly in the abdominal aorta where the cracks and fissures were bordered by proliferating spindle cells (Fig. 17).

3. Coronary arteries

In the coronary arteries the mildest of the lesions noted was interruption of the internal elastic lamina, usually in several places, producing irregular fragmentation but no obvious fraying or reduplication. The artery shown (Figs. 18 & 19) illustrates an early example of this. Although interruptions of this degree are occasionally noted in human tissues, none was seen in any of the control pigs (Fig. 20). This was evidently followed by radially oriented fissures which penetrated the muscular media eventually as far as the adventitia (Figs. 21 and 22). Small dissecting haemorrhages, either within the media or between the media and the adventitia, appeared to be sequelae of such fissures and were the earliest lesions to be visible grossly (Fig. 23). In 3 animals it was assumed that such dissections became massive, compressing the media eccentrically within the still intact adventitia, thereby occluding the lumen and producing myocardial infarction (Fig. 12). Figures 24 and 25 show sections from a similar lesion (Fig. 13) at some distance from the occlusion. The line of cleavage in each case was immediately internal to the

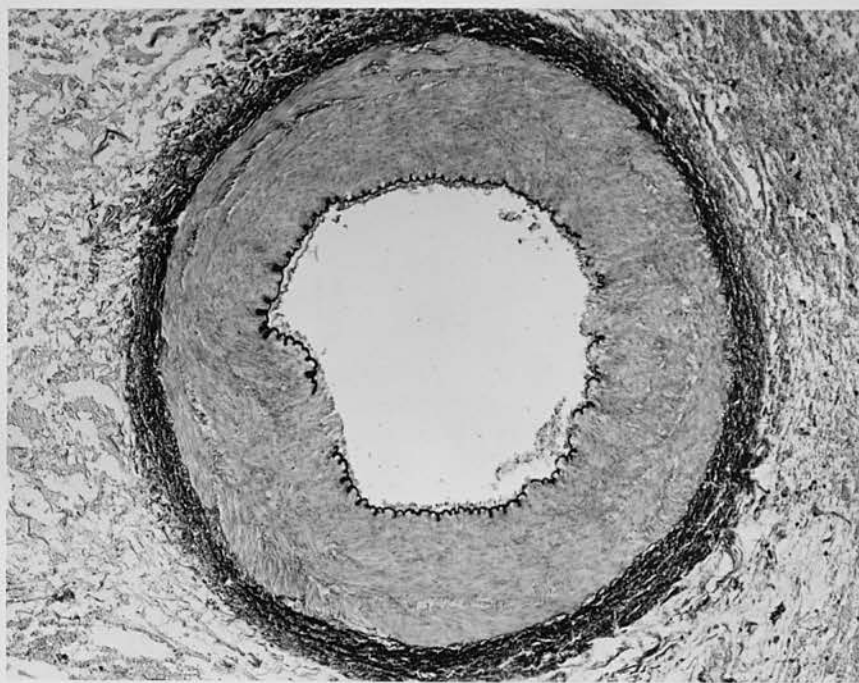


Fig. 18. Coronary artery. The internal elastic lamina is fragmented in several places. Verhoeff elastic stain. x 80.

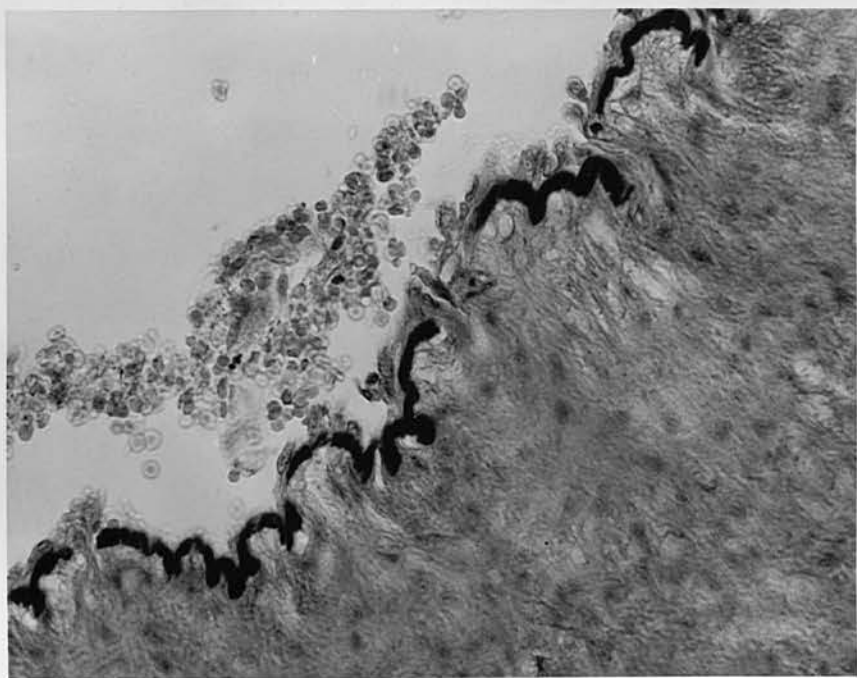


Fig. 19. Coronary artery. A higher power detail of Figure 18 shows the internal elastic lamina. x 600.



Fig. 20. Coronary artery, control pig. The internal elastic lamina is continuous. Verhoeff elastic stain. x 75.

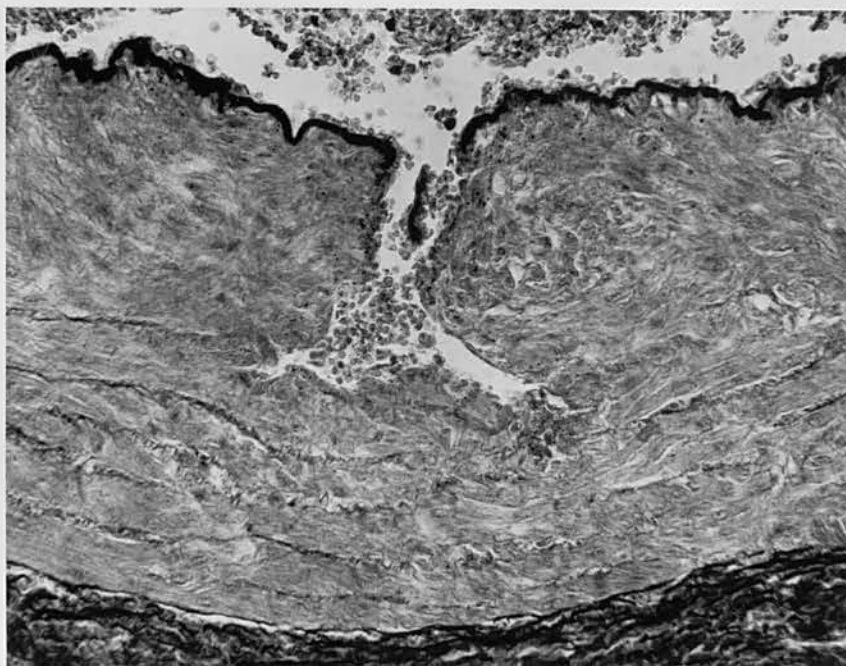


Fig. 21. Coronary artery. Longitudinal section shows a fissure penetrating radially into the media at the site of a breach in the internal elastic lamina. Verhoeff elastic stain. x 300.

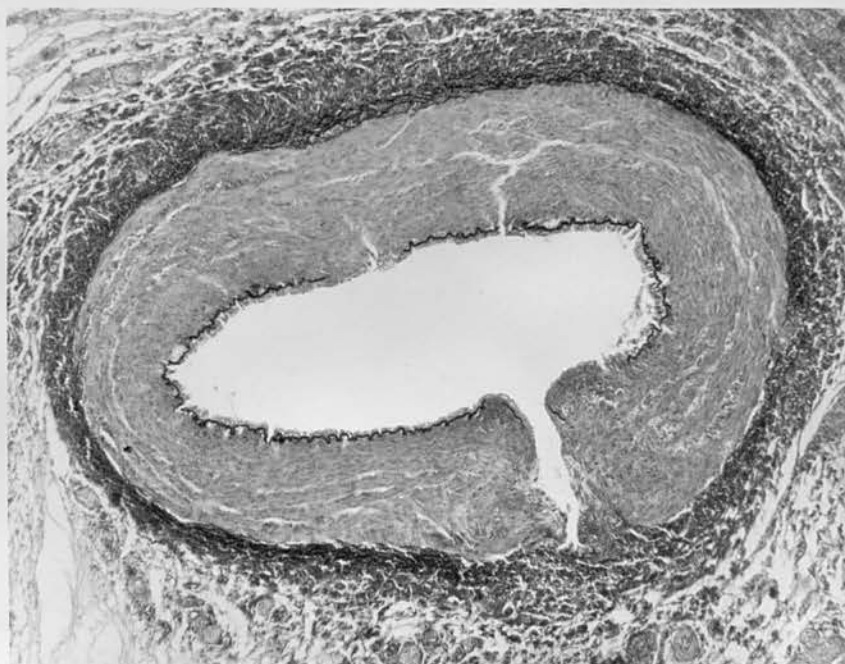


Fig. 22. Coronary artery. The internal elastic lamina is deficient in several areas, with a wide fissure penetrating to the adventitia at one such site. Verhoeff elastic stain. x 100.

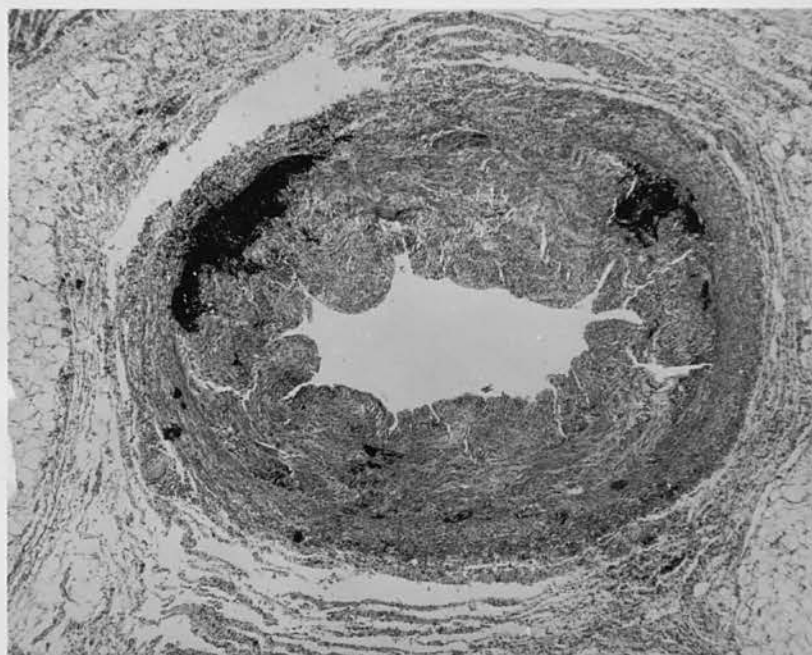


Fig. 23. Coronary artery. Numerous small fissures penetrate the mediate. There are two small intramural haemorrhages, limited externally by the adventitia. Masson's trichrome stain. x 80.

external elastic lamina. The infarct in one of these animals ruptured. More frequently (in 8 animals) dissection was followed by complete rupture of the vessel wall, producing large sub-epicardial or myocardial haemorrhages (Figs. 7 & 26), which were always complicated by haemopericardium.

4. Other vessels

Lesions of the internal elastic lamina were seen in muscular arteries of all dimensions in both visceral and musculoskeletal distribution (Fig. 27). The renal artery illustrated (Fig. 28) not only shows interruption but also abnormal alignment of the fragments of the lamina. This was associated with early medial fissuring and focal medial haemorrhages, but in no instance were more advanced lesions found. A renal vessel of similar size from a control animal is shown for comparison (Fig. 29). In established copper deficiency, a noticeable external feature of affected swine was the presence of large subcutaneous haematomas overlying pressure points, particularly in the limbs. No ruptured arteries have been identified in connection with these, although neighbouring vessels showed less advanced lesions.

In addition to the lesions of internal elastic lamina and tunica media, very interesting changes were not infrequently observed in the intima of small vessels. The innermost cells of the media tended to protrude through gaps in the internal elastic lamina, elevating the endothelium (Fig. 30), or everting the edges of the broken membrane (Fig. 31). In one coronary artery a well formed plaque, containing muscle cells, collagen, and some elastin was noted (Fig. 32). No stainable fat was ever seen in these intimal lesions.

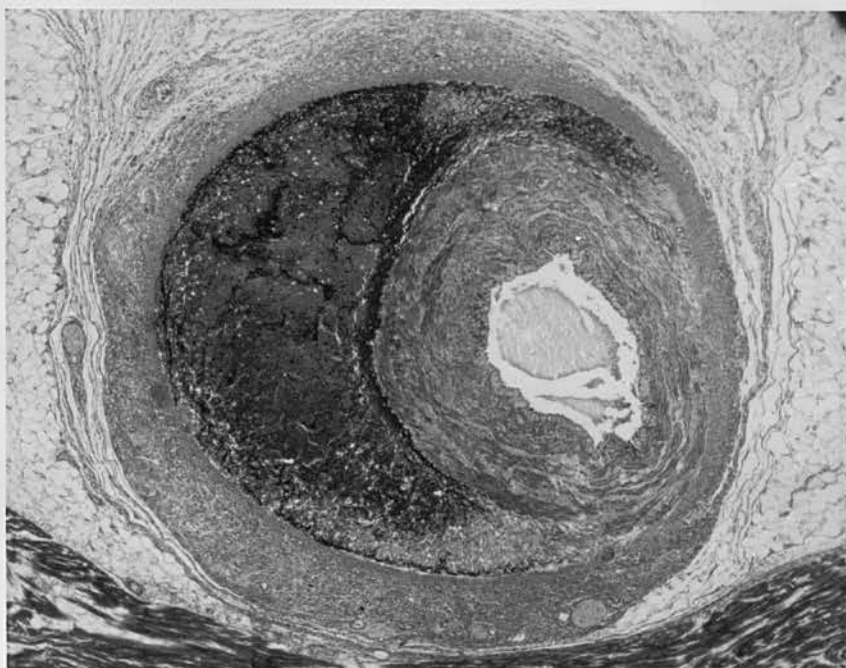


Fig. 24. Coronary artery. A large haemorrhage is contained within the adventitia and is displacing the medial tube to one side. Masson's trichrome stain. x 50.

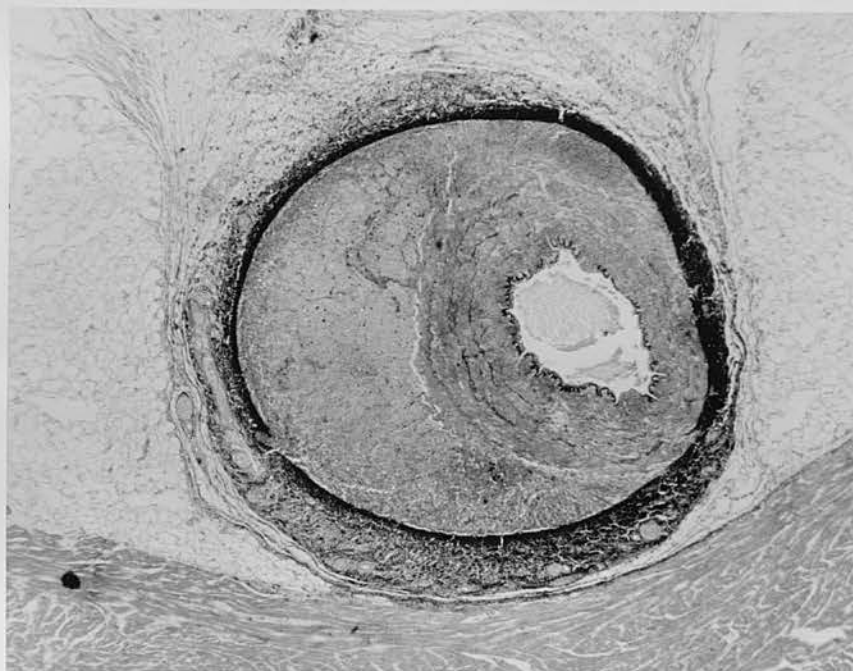


Fig. 25. Coronary artery. The intact external elastic lamina of the adventitia contains the dissecting haemorrhage. Verhoeff elastic stain. x 35.

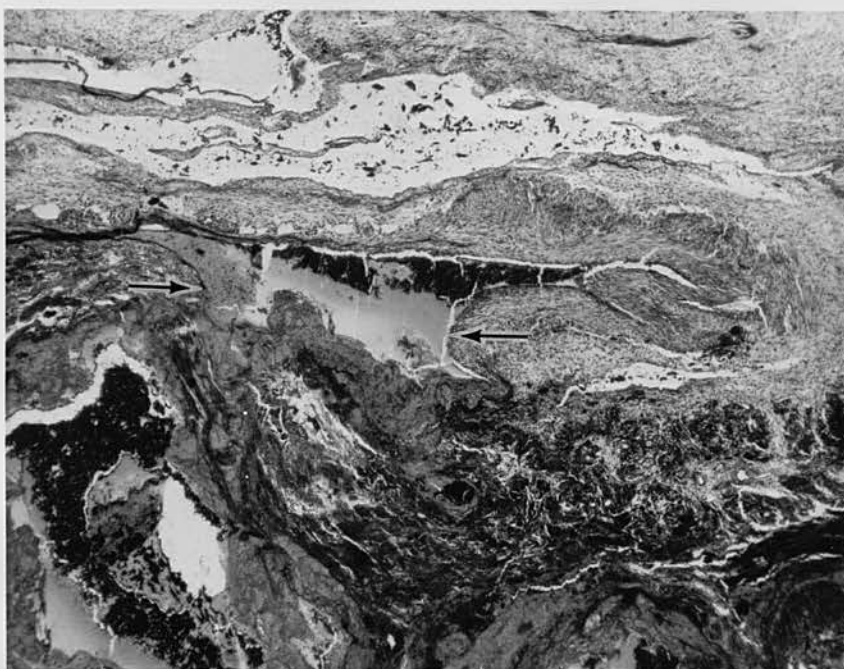


Fig. 26. Coronary artery. A longitudinal section exhibits complete rupture of the wall, indicated by arrows, and massive haemorrhage. Masson's trichrome stain. x 25.

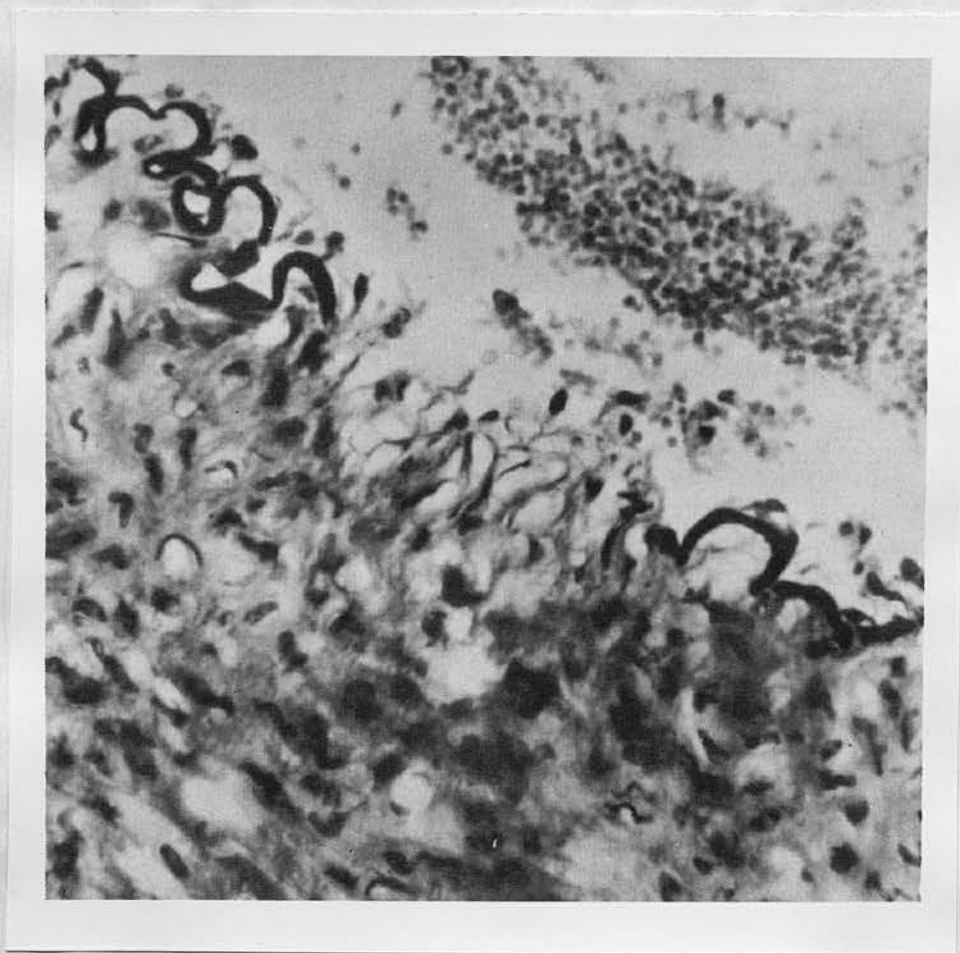


Fig. 27. Splenic artery, showing break in internal elastic membrane. Endothelium is discontinuous. Verhoeff-van Geison stain. x 300.

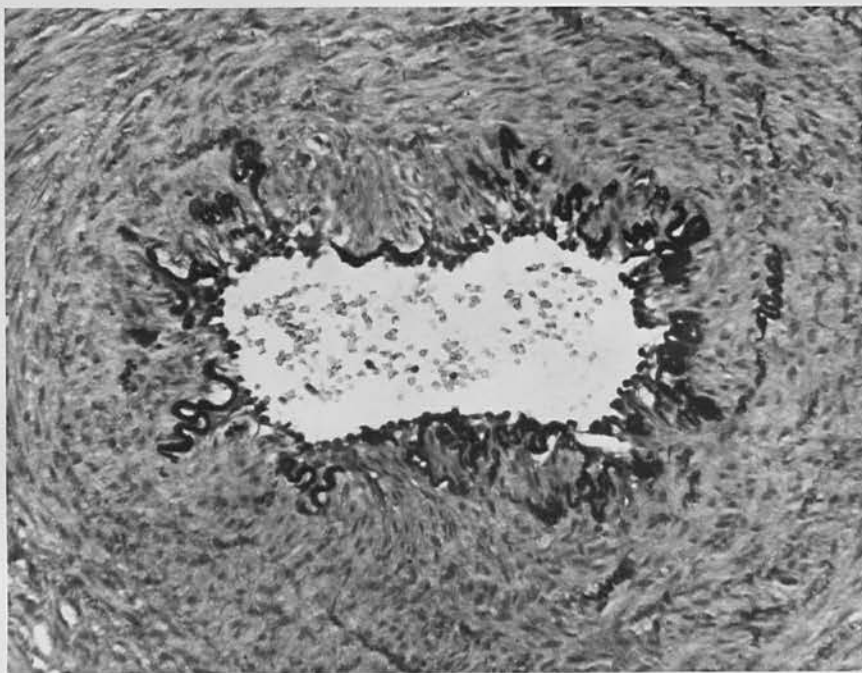


Fig. 28. Renal artery. The internal elastic lamina is fragmented. Verhoeff elastic stain. x 270.

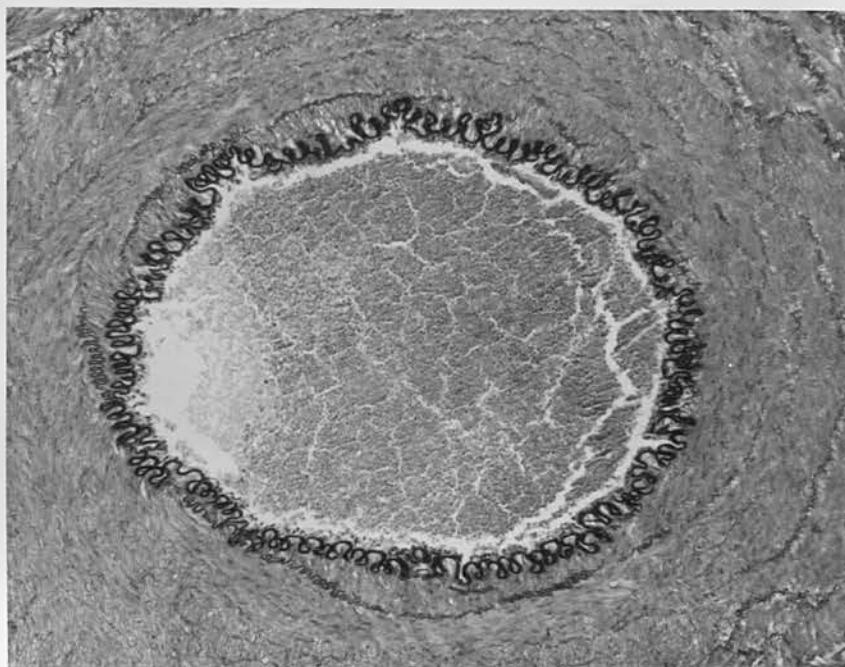


Fig. 29. Renal artery, control pig. The internal elastic lamina is unbroken. Verhoeff elastic stain. x 130.

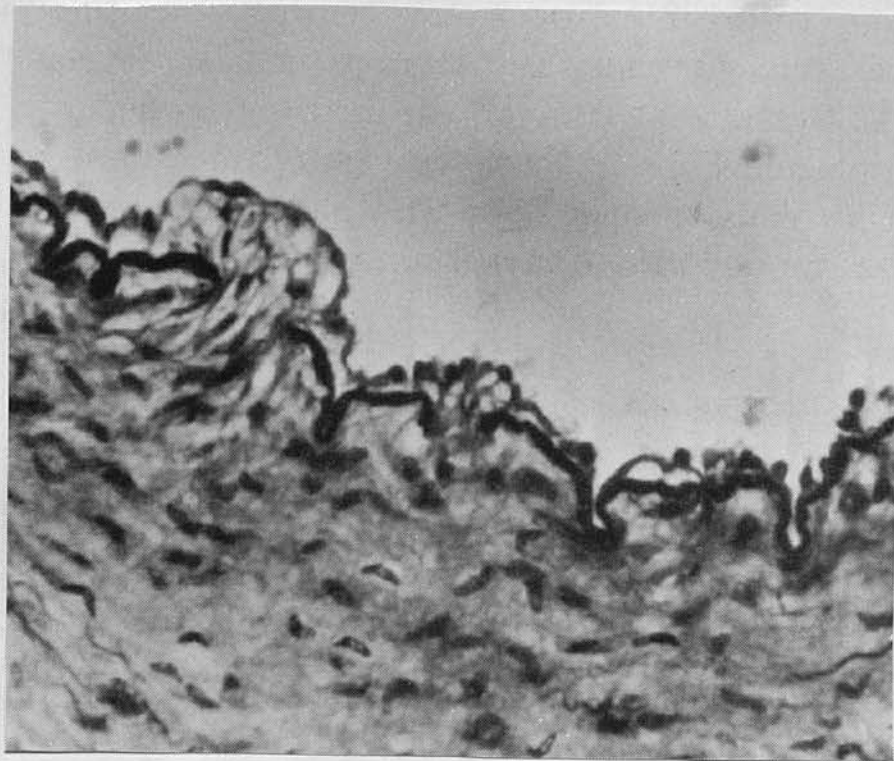


Fig. 30. Coronary artery, showing elevation of endothelium by vacuolar spaces and by cells of media that protrude through breaks in internal elastic membrane. Verhoeff-van Gieson stain: x 300.



Fig. 31. Splenic artery, showing protrusion of medial cells beyond everted edges of broken internal elastic membrane. Endothelium poorly preserved. Verhoeff-van Gieson stain. x 300.

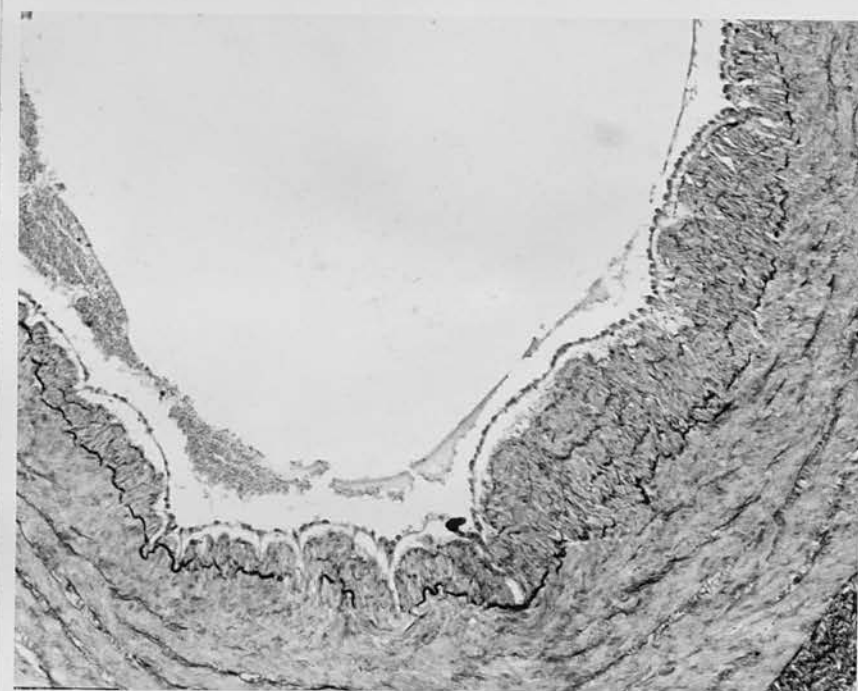


Fig. 32. Coronary artery. A segment of the inner part of the wall shows a fragmented internal elastic lamina and thickening of the intima by muscle fibres and fragments of elastin. Verhoeff elastic stain. x 150.

Discussion

The good correlation between the degree of anaemia and the relative heart weights in this experiment was consistent with the hypothesis that the cardiac hypertrophy was a response to increased cardiac output consequent upon anaemia. Cardiac hypertrophy in copper-deficient swine has been reported previously, and a greater degree of hypertrophy has been noted in copper-deficient than in iron-deficient animals with comparable degrees of anaemia (Gubler et al., 1957). The lesser degree of hypertrophy in the iron-deficient animals in that experiment is unexplained. The serous effusions were probably due to cardiac failure. These occurred in the copper-deficient and in the iron-deficient groups alike.

Cardiac rupture occurred only in the copper-deficient groups. In some instances this was a consequence of coronary vascular lesions leading to myocardial infarction. In 6 instances there was rupture of papillary muscles that could not be attributed to vascular lesions. Rupture of the atria in 2 instances was almost certainly coincident with a marked regurgitation through atrio-ventricular valves following rupture of papillary muscles. These events occurred in some of the largest hearts. Dilatation and increased stroke volume may have placed an exceptional strain upon the papillary muscles. However, the unusual friability of the myocardium, demonstrable at necropsy, was believed to indicate an underlying defect in muscular or connective tissue structure. The lack of a histologically demonstrable antecedent lesion leaves the exact site of the defect undisclosed at present. Additional work undertaken subsequent to this experiment, in conjunction with A. N. D'Agostino, involving electron microscopic examination of

the cardiac papillary muscles of copper-deficient pigs, also failed to demonstrate a structural lesion.

Histologic study of the aorta and other arteries came closer to providing an explanation of the vascular ruptures. That significant histologic defects exist in the vessels prior to rupture has been amply demonstrated. The most conspicuous structural defects appeared in the elastic tissue, suggesting that the basis for the histologic defects might reside in this component. However, it was not clear whether these abnormalities were primary or whether a rupture of elastic laminae was the consequence of altered mechanical properties of other supportive components of the vascular wall. The importance of the internal elastic lamina in maintaining the integrity of a vessel wall has been demonstrated by Glynn (1940). Human cerebral arteries from the circle of Willis were inflated with air, using pressures of up to 600 mm of mercury. Defects in the muscular media, either natural or produced artificially, had no effect on the form of the vessel, provided that the internal elastic lamina remained intact. More recently, Molnar et al., (1962) demonstrated obvious aneurysmal expansion of dog femoral and rabbit ear arteries, following disappearance of the internal elastic membranes when highly purified trypsin-free elastase was injected intra-arterially "in vivo".

It is probable that the entire sequence of events now reported in the coronary vessels was the result of mechanical disruption by the blood flow following focal rupture of the internal elastic lamina. Although medial fissures and intramural haemorrhages

occurred in the abdominal aorta, extensive dissection or external rupture were not observed as complications. This may have been a reflection on the multiplicity of laminae in the aorta as compared with the single lamina of the coronary arteries. A different mechanism is postulated in explanation of the large tears which occurred in the first parts of the aorta and pulmonary trunk. By Laplace's law, tension in a cylindrical tube varies as its radius and would therefore be maximal in the proximal segments of these vessels. A combination of maximal tension and malacia of the wall was apparently sufficient to produce a direct "blowout." These large ruptures never dissected more than short distances along the vessel, and in no instance could continuity be established between an aortic and a coronary lesion.

Similar aortic lesions have been described in human cases of dissecting aneurysm, including Marfan's syndrome, but whenever the coronary vessels were involved, it was the result of proximal dissection in continuity. No similar coronary vascular lesions have yet been reported in experimental lathyrisms in any species, although an elastic fibre defect has been described in lathyrotic rat arteries (Walker, 1957).

The evidence that the cardiovascular lesions were due to copper deficiency may be summarized as follows: (1) The lesions were present in the animals fed a diet deficient in copper; (2) control animals fed the same diet but supplemented with copper did not develop such lesions; (3) restriction of the dietary intake of the control animals to the amount ingested by the copper-deficient animals did not result in the appearance of lesions in

the cardiovascular system of the control pigs; (4) the addition of 7 trace elements other than copper, 12 water-soluble vitamins and 4 fat-soluble vitamins to the diet of the copper-deficient pigs did not prevent the development of lesions; and (5) these lesions, excluding cardiac hypertrophy, were not observed in iron-deficient pigs with anaemia comparable in severity and duration to the anaemia in copper-deficient pigs. Thus, it seemed reasonable to conclude that the vascular lesions resulted from a dietary deficiency of copper.

Having established the fragility of the cardiovascular system in copper deficiency, and obtained preliminary evidence that the elastic tissue was defective, it was then considered that quantitation and analysis of this fragility, particularly that of the aorta, might well form the basis of the next experiment.

Experiment 2.

The mechanical properties of the intact aorta

The observed fragility of vessels, particularly the aorta, suggested the next experiment in this study. Analysis of aortic load-extension curves had been used to demonstrate the interplay of elastic properties of different mural components (Roy, 1880; Hallock and Benson, 1937; Krafka, 1937; Hass, 1942; Remington, 1955; Roach and Burton, 1957; Bergel, 1961; Speckman and Ringer, 1964; Wolinsky and Glagov, 1964; Learoyd and Taylor, 1966). The application of this technique to the aortas of copper-deficient swine might therefore help to define more accurately the site of the vascular lesion. Loops of aorta were stretched diametrically to give static linear load-extension diagrams.

Experimental

Twenty-two animals from two litters of purebred Yorkshire and Duroc-Yorkshire cross swine were received from a local breeder at 2 days of age. They all received the basal milk diet supplemented with iron and other minerals. Nine pigs were fed copper and thus acted as controls to the remaining 13, which were copper-deficient.

Seven of the copper-deficient pigs died at from 61 to 88 days. The remaining 6 deficient and the 9 control animals, were killed by exsanguination under anaesthesia at from 71 to 101 days. Segments from the lower third of the descending thoracic aorta, between branches, were cut into rings with a system of razor blades rigidly mounted 0.4 cm apart. The rings were stored in normal saline at 4°C until used. Stretching was always performed within 48 hours.

After the adventitia was stripped, mean values for width and thickness of each ring were measured with a calibrated stereomicroscope from which the cross-sectional area, A , was calculated.

The stretching apparatus is shown in Figure 33. A reversible motor (A), capable of regulation by a two-tube control box at different relative speeds from 1 to 10, drives a lead screw (B) and a 10-turn potentiometer (C). The lead screw moves a block, bearing the lower hook (D), so that one complete turn of the screw moves the hook through 1 mm. The potentiometer drive is mediated through a belt and a system of pulleys (E) giving a selection of stretch ranges from 2 to 10 cm. Two microswitches (F) provide a safety mechanism at the upper and lower limits of the stretching block. The upper hook (G) is threaded onto the probe of a transducer (H) (Statham model G1). The models used in this experiment have force ranges of 8 and 80 ounces. Excitation of both the transducer and the potentiometer is at 12 volts d.c. by a voltage regulator (Video model SR-200EP). The transducer current, measuring applied load, and the potentiometer current, measuring extension in the specimen, are fed into the Y and X axes respectively of an X-Y recorder (Moseley model 3S). In response to the electrical data applied to the two axes, a pen traces a cartesian coordinate graph of the relationship between the input signals, which in this experiment are equivalent to stretch distance and load developed. The X and Y ranges can be variably adjusted so that the entire spans of the two axes may be used. Both hooks are enclosed in a glass jacket (I), providing an immersion bath for the specimen.

Each ring was suspended in normal saline between the two hooks, which were initially in contact. Stretching was performed at 21°C.

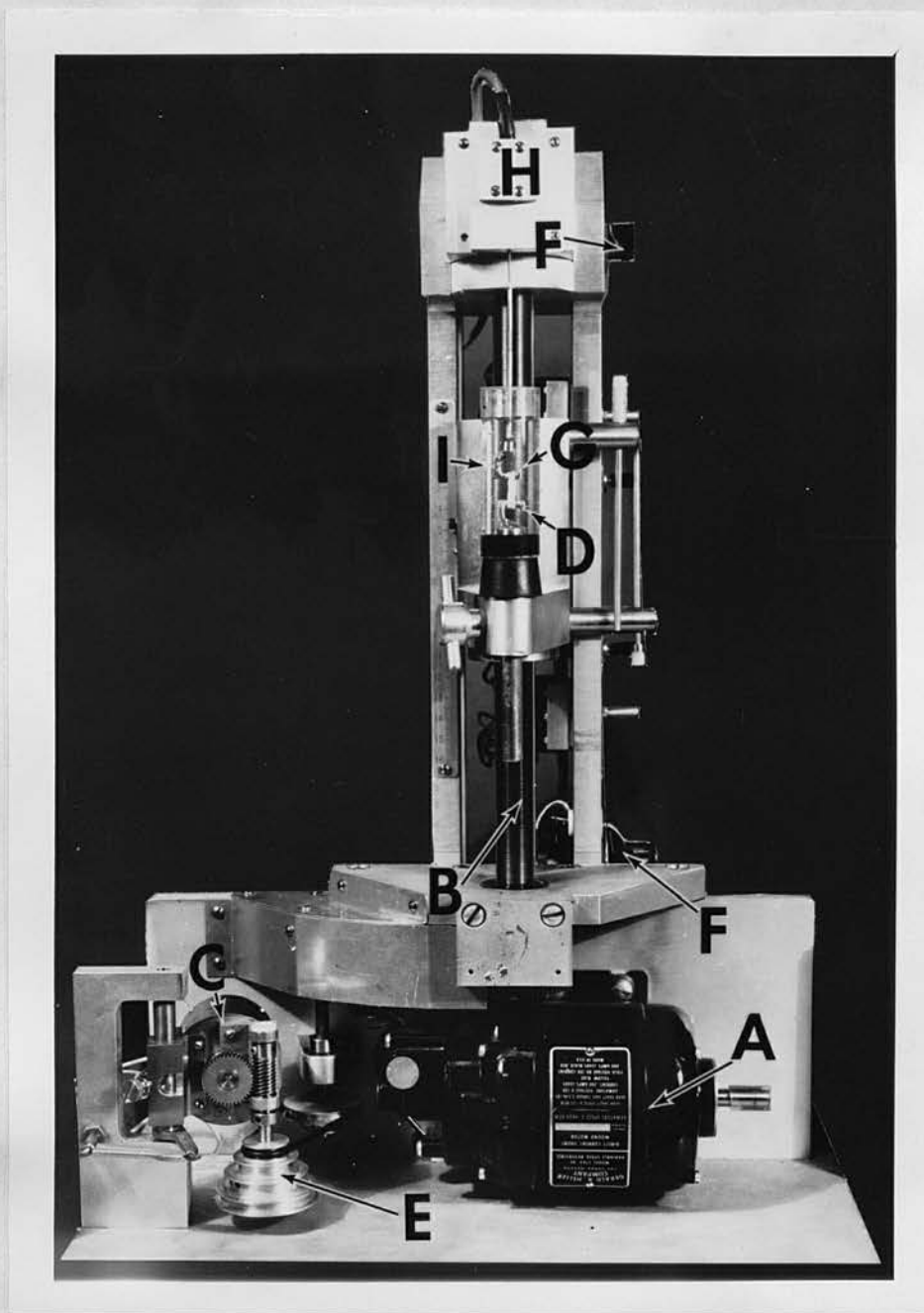


Fig. 33. The stretching apparatus.

A, reversible motor; B, lead screw; C, potentiometer;
 D, lower hook; E, pulleys; F, microswitches;
 G, upper hook; H, transducer; I, glass jacket.

A uniform speed of 3 mm per minute was maintained. Using the 8-ounce transducer, a load of 100 to 150 g was developed before the motor was reversed, allowing relaxation to occur and thereby tracing a hysteresis curve. After a short recovery interval this was repeated at least once, and occasionally several times. With the 80-ounce transducer in position the rings were then stretched to the breaking point.

Results

A difference in mural thickness was quite obvious between control and copper-deficient animals. The mean values are given in Table 6.

The reversible load-extension curves described hysteresis loops, the areas within which were always noticeably greater with the initial "conditioning" stretch (Figs. 34 & 35). Successive stretching produced a gradually diminishing increase in length for a given load, but all rings returned to the original length, indicating negligible viscous flow. The curve pattern was typically biphasic. The first phase, of low gradient, indicated a relatively substantial increase in length with increasing load. This phase passed smoothly into the second, of much higher gradient, in which a considerable increase of load produced little further elongation. A breaking stretch curve was predominantly linear in its second phase (Fig. 36). Before rupture the specimen yielded, exhibiting some plasticity, but in many of the control rings this was hardly noticeable and the break occurred suddenly, transversely across one limb. In none of the rings did rupture occur in the region of contact with a hook. A yield phase was

Table 6.

Mechanical Properties of Aortae from Control and Copper-Deficient Swine

<u>Group</u>	<u>No. of pigs</u>	<u>Aortic mural thickness</u>	<u>cm</u>	<u>Ultimate strength</u> <u>kg/cm²</u>	<u>Stretch modulus</u>	
					<u>1st phase</u>	<u>2nd phase</u>
					<u>kg/cm²/100% elongation</u>	
Control	9	0.13 ± 0.03		14.9 ± 2.7	1.23 ± 0.24	95.6 ± 27.0
Copper-deficient	13	0.23 ± 0.04		3.4 ± 1.0	0.31 ± 0.10	28.0 ± 9.0

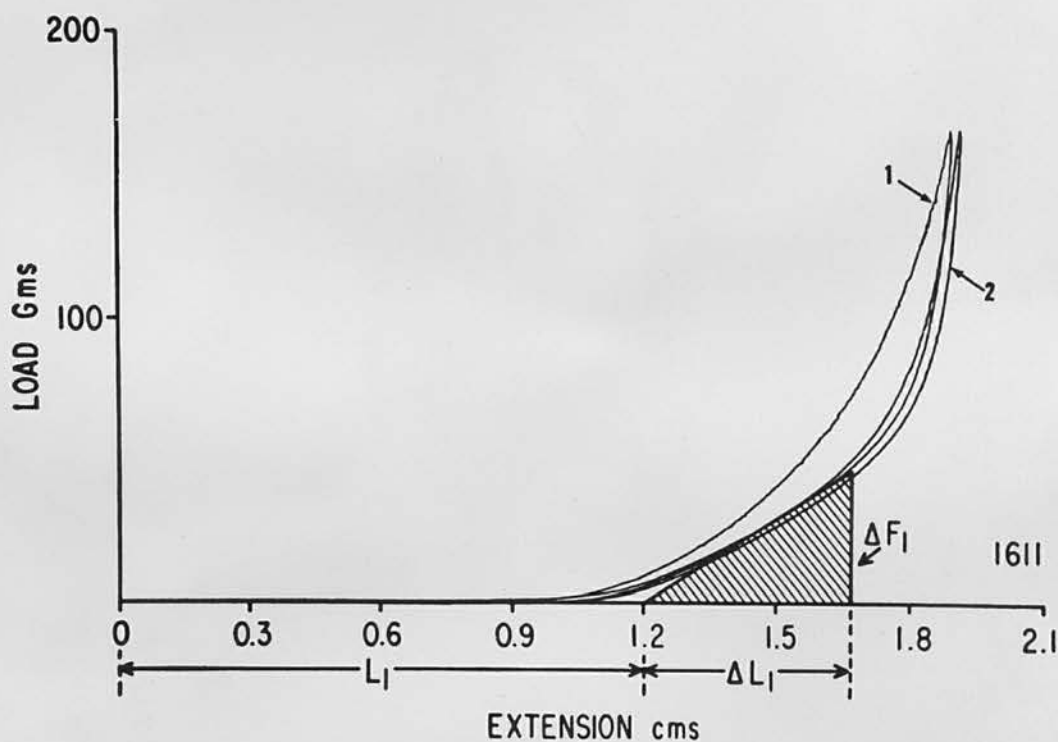


Fig. 34. Load-extension diagrams of an aortic loop from a control pig, showing the initial stretch, curve 1, and a subsequent stretch, curve 2. The hatched triangle illustrates the method of derivation of a tangential modulus to the first phase of curve 2. An 8-ounce transducer was used.

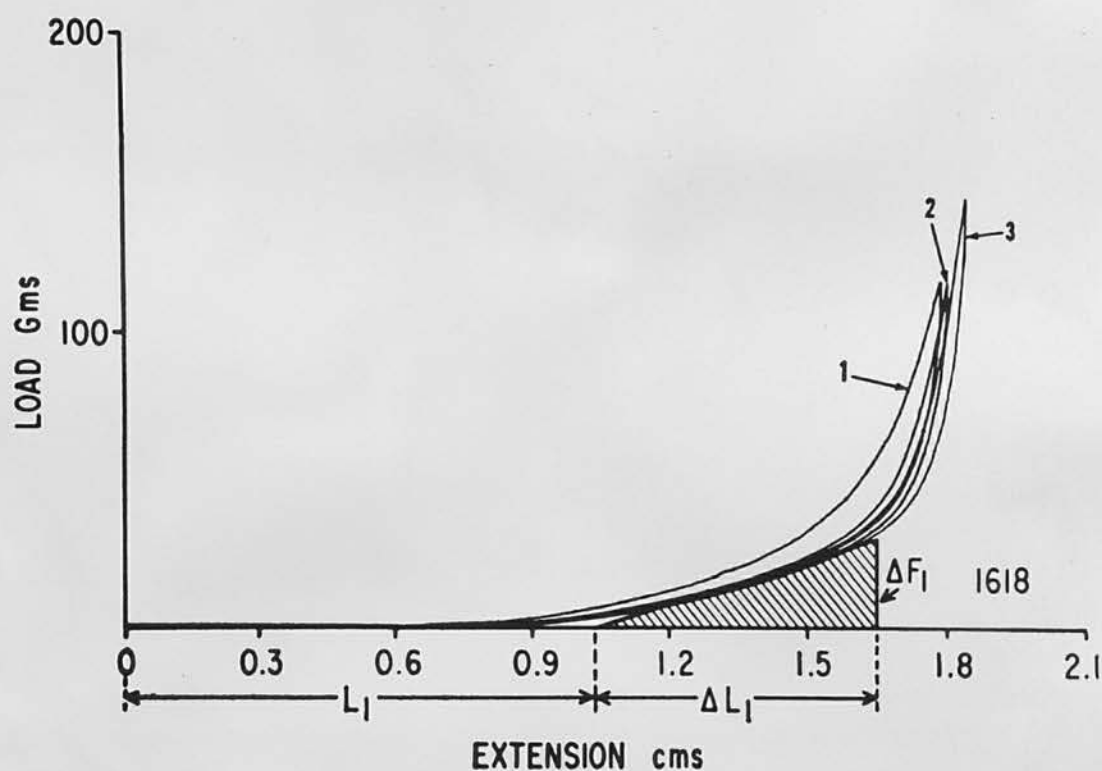


Fig. 35. Load-extension diagrams of an aortic loop from a copper-deficient pig, showing the initial stretch, curve 1, and two subsequent stretches, curves 2 and 3. An 8-ounce transducer was used.

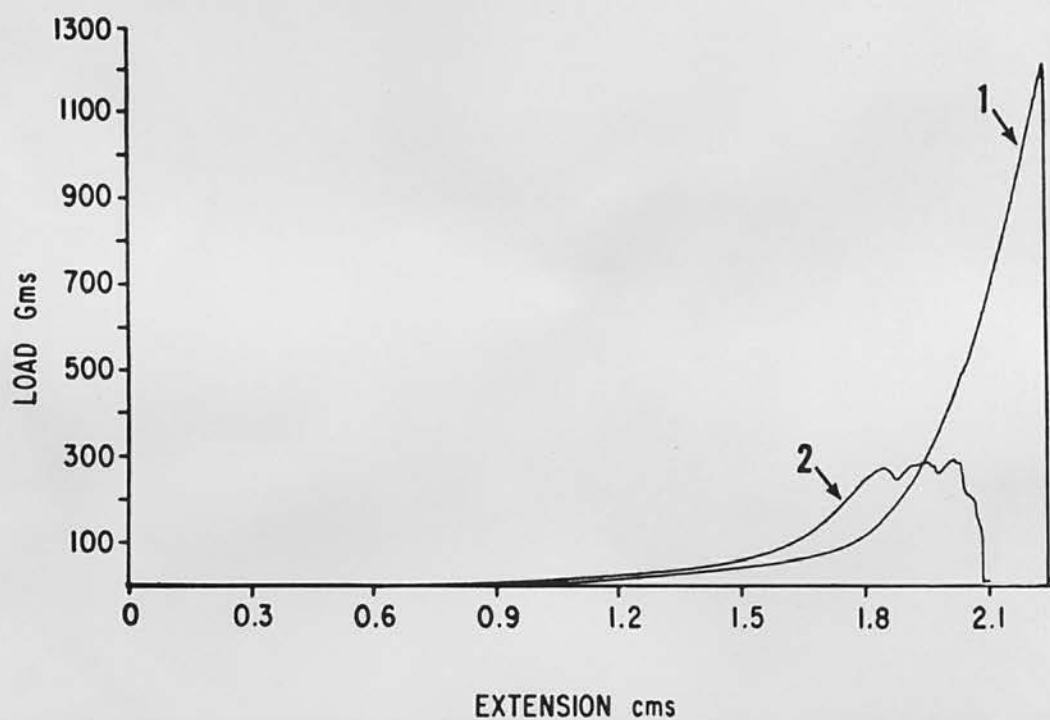


Fig. 36. Load-extension diagrams illustrating the mode of rupture of aortic loops from a control pig, curve 1, and a copper-deficient pig, curve 2. An 80-ounce transducer was used.

quite characteristic of the copper-deficient rings, producing a smooth hump rather than a sharp peak (Fig. 36). Rupture took place gradually, beginning at the intimal surface and winding obliquely around the two hooks, so that the final specimen had the configuration of a flattened helix (Fig. 37). This behaviour manifested itself graphically as a stepwise diminution in tension with progressive extension.

In order to characterize mechanical properties, stretch moduli were derived for the best tangents to the two phases of the extension curve (Figs. 34, 35, 38 & 39). The initial length (L) of each loop, equivalent to half the circumference, was measured as the distance between the supporting edges of the hooks at the intersection of tangent and abscissa. The increment in length ΔL , produced an equivalent increment in tension, ΔF . The modulus, M, was given by the formula,

$$M = \frac{\Delta F/2A}{\Delta L/L} \text{ gm/cm}^2/\text{100\% elongation}$$

Ultimate strength was calculated as the maximal tension developed per unit cross-sectional area. In the control animals this corresponded to the point of rupture and in copper-deficient animals to the maximum of the curve, where rupture had begun but was incomplete. These values are recorded in Table 6 and show a 3- to 4-fold reduction in both moduli and in ultimate strength in the deficient as compared with the control aortae.



Fig. 37. Aortic rings after extension to breaking point.
A, from a control pig, shows a simple rupture;
B, from a copper-deficient pig, shows a long
oblique rupture, coursing helically around the
aortic wall.

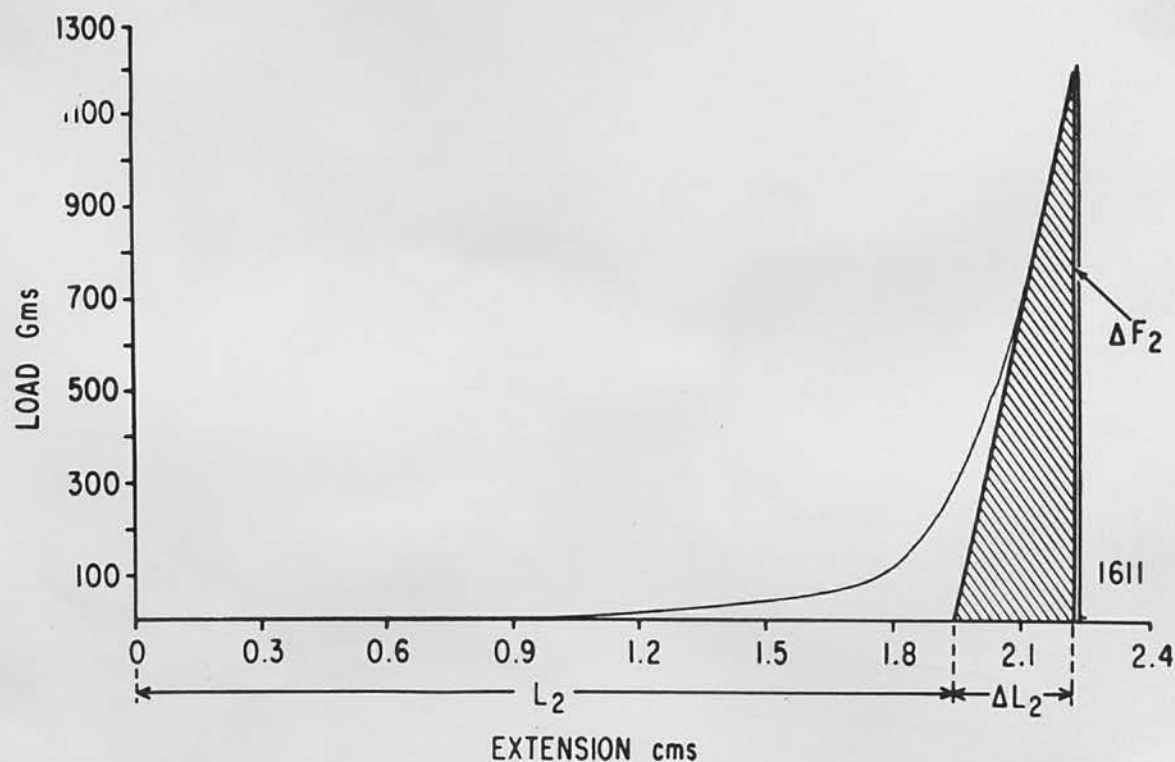


Fig. 38. Load-extension diagram of an aortic loop from a control pig (curve 1 of Figure 36), illustrating the derivation of a tangential modulus to the second phase of the curve. An 80-ounce transducer was used.

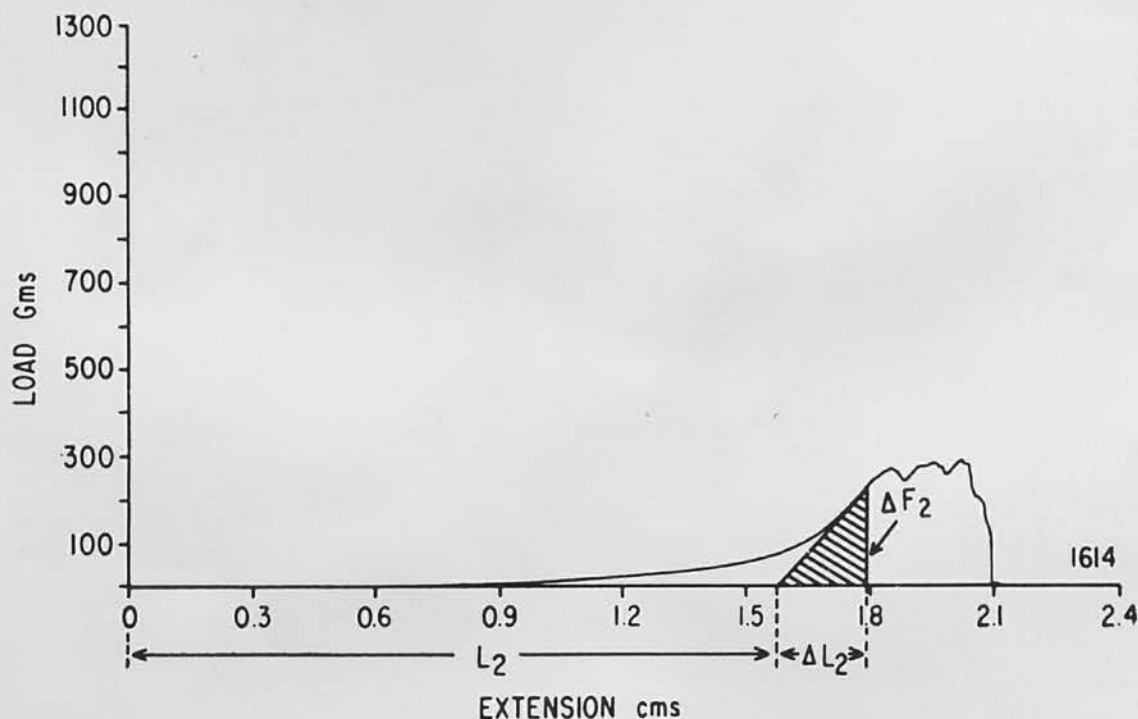


Fig. 39. Load-extension diagram of an aortic loop from a copper-deficient pig, stretched to the breaking point; 80-ounce transducer. As in Figure 38, the hatched triangle is bounded by the tangent to the second straight phase of the curve and the lines, indicating corresponding increments of length and force for this phase, that are used to calculate the stretch modulus.

Discussion

Most homogeneous materials become progressively more extensible with increasing load, and a load-extension graph assumes a course parallel to the extension axis. When aorta is stretched there is an almost linear relationship between load and extension initially, but thereafter progressive loading produces little further extension, and the graph curves toward the load axis. As Burton (1954) pointed out, this may be explained by assuming that the two main tensile components of aorta, elastin and collagen, function to some extent independently. Elastin is a rubber-like, extensible material, with a stress-strain response similar to that of the first phase of stretched aorta. Collagen is virtually inextensible and its stress-strain graph approximates to the second phase of that of the aorta. One must postulate that initial loading stresses elastin fibres only, until the unstretched length of relatively slack collagen fibres has been exceeded, after which the load is borne almost entirely by collagen, until rupture occurs.

Laplace's law states that tangential tension in a cylindrical vessel is the product of the contained pressure and the radius. The tension developed in pig thoracic aorta "in vivo", assuming a systolic pressure of 120 mm of mercury, would be about 50 gm. This falls within the first phase of the normal load-extension curve described in this experiment, in which tension is maintained predominantly by the medial elastic fibres.

The stretch moduli calculated for the aorta may be adjusted to characterize elastin and collagen. The proportions of elastin and collagen in normal pig aortic media have been calculated by

Weissman (1963). Estimating hydroxyproline content, he derived a value for collagen of 17.3 per cent of dry, fat-free weight. The elastin residue was calculated, following triple autoclaving of dried, alcohol-ether extracted tissue, as 68.7 per cent. These results agree substantially with those of Neuman and Logan (1950). Assuming these values, the adjusted moduli are 1.78 kg per sq.cm in the first phase (elastin) and 530 kg per sq.cm in the second phase (collagen).

The corresponding figures for aortic collagen and elastin in copper-deficient pigs are 18.0% and 60.1% respectively (Weissman, 1963). Moduli based on these values are 0.5 and 14.0 kg per sq.cm for the first and second phases of the stretch curve, respectively.

Whichever method is used to calculate appropriate values for the extension moduli, it is apparent that for both phases there is a three- to four-fold reduction in the moduli of the copper-deficient aortas as compared with the controls. In the case of the second phase of extension, this is further emphasised by a similar reduction in aortic ultimate strength, also predominantly a measure of collagen function. It might be inferred, therefore, that both elastin and collagen in the aorta are affected by copper deficiency.

At this point I should digress briefly to summarize the relationship of the chief structural components of the aorta. The medial elastic tissue forms fenestrated, circumferential lamellae (Hass, 1942). These are bound together by muscle cells (Keech, 1960; Pease and Paul, 1960; Karrer, 1961). The collagen fibres

form loosely arranged bundles lying parallel and close to the elastic lamellae and may pass through the fenestrations, but as yet there have been no reports of direct connections between collagen and either elastin or muscle cells. Mucopolysaccharide-protein complexes form the ground substance. When discussing the roles of collagen and elastin in the mechanical properties of the aorta, it has been customary to regard them as acting in parallel and presumably independently (Burton, 1954). If this were so, it would require that both elastin and collagen were affected by copper deficiency. If, however, their tensile properties were not independent, but mutually inter-related, a lesion of one component could affect the behaviour of the other and it would not be necessary to postulate lesions of both.

Thus, if collagen fibrils and fibres were normal in these aortas, their function in a stretch resisting framework might be seriously impaired if the elastin were defective. With multiple variables, further work on the elucidation of the nature of the mechanical defect in copper-deficient pig aortas required the isolation and testing of each component individually.

Experiment 3

The mechanical properties of isolated aortic elastin

In order to eliminate, as far as possible, mutual interaction of the various components of the aortic wall in its response to stretching, methods were sought for the isolation of an individual component. A technique for the isolation of aortic elastin, using concentrated formic acid, had been described by Hass (1942). Subsequently Ayer et al. (1958) showed that an aortic elastin residue produced in this way, was free of other tissue elements and that its elastic recoil was unimpaired.

The stretching technique described in Experiment 2 was performed on the elastin residues obtained from the aortas of copper-deficient and control swine, by formic acid treatment.

Most of the technical work in this experiment was done by D. A. Kimball, during his tenure of a Research Traineeship in the Department of Pathology, University of Utah College of Medicine.

Experimental

As a preliminary to the main experiment the effect of formic acid on the weight and tensile strength of normal swine aorta was determined. Loops of aorta, obtained from mature pigs at the slaughter house, were cut from a 15 cm segment of the descending thoracic aorta. Thirty-one loops were desiccated, weighed, and treated with formic acid at 45°C for periods of 6, 24, 48, 96, 192, and 360 hours. Figure 40 shows the reduction in weight after formic acid extraction for varying periods of time. The weight of the residue dropped rapidly during the first 6 hours, then reached

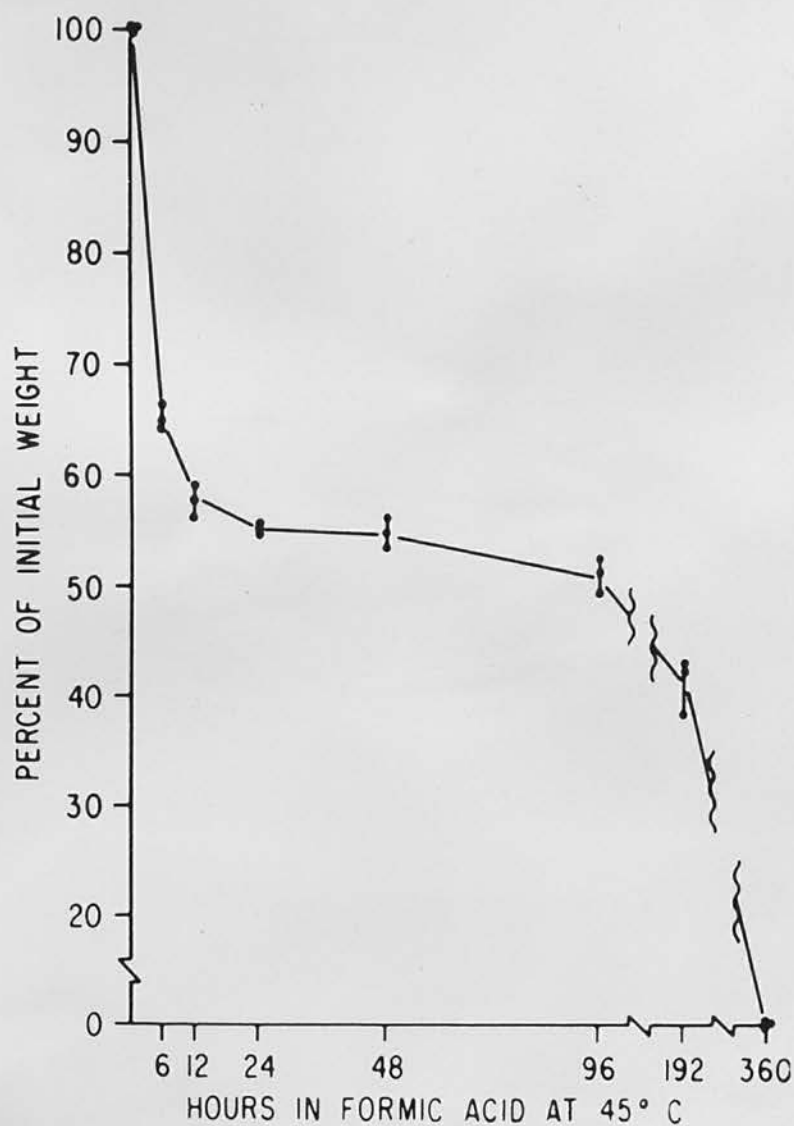


Fig. 40. Decrease in aortic residue after formic acid extraction.

Each point represents one determination.

a plateau which decreased only slightly between 12 and 96 hours. Finally, the entire residue went into solution by 360 hours. Figure 41 shows the tensile strength of the residue after varying periods of extraction. This also dropped rapidly during the first 6 hours, when it reached a plateau at about 10 kg per sq.cm and then slowly decreased after 96 hours. Both the tensile strength and the weight remained relatively constant during the period between 24 and 96 hours. Therefore, 36 hours was selected as the optimum time for extraction in the remaining experiment.

A total of 33 pigs from several litters of purebred Yorkshire swine were obtained at ages of 4 to 6 days from a local breeder. They were divided into a control group of 12, a copper-deficient group of 14, and a second copper-deficient group of 7 swine, which also received the mineral and vitamin supplements. All the animals either died or were killed by exsanguination under anaesthesia between 71 and 102 days of age.

At autopsy, transverse rings of aorta 0.4 cm wide were cut by razor blades from the descending thoracic aorta. These were cleansed of blood and surrounding tissue and rinsed in saline. The rings were desiccated over P_2O_5 at a reduced pressure for at least 4 days and stored in the desiccator until treatment with formic acid. Each ring was weighed dry and then placed in a sealed bottle containing concentrated formic acid (88%) in a water bath at 45°C for 36 hours. One ml of formic acid was used for every 5 mg of dry tissue. Following extraction, the rings were washed in running water for 24 hours. The width and thickness of each ring was measured with a calibrated stereomicroscope, and extension to breaking point was performed without preliminary hysteresis.

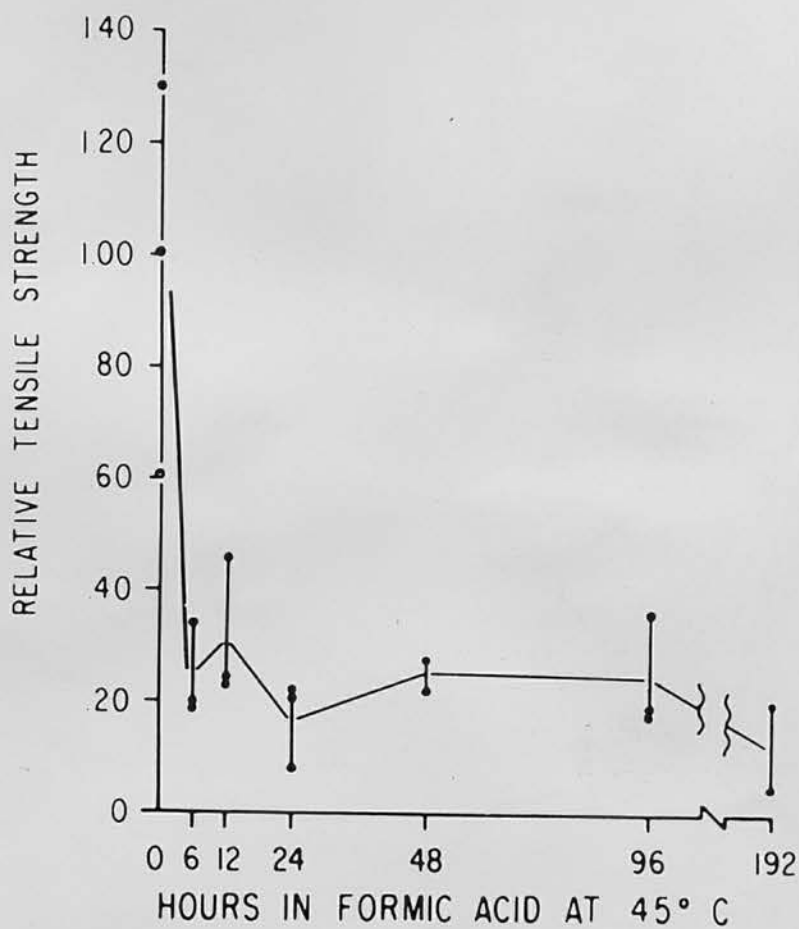


Fig. 41. Decrease in aortic tensile strength during formic acid extraction. Each point represents one determination.

Following rupture, the rings were again desiccated over P_2O_5 at a reduced pressure for at least 4 days and weighed, so that a comparison of weights before and after formic acid extraction could be made.

Results

During formic acid extraction it was observed that the aortic rings became gelatinous and swollen to about twice normal size. They returned to their former shape upon washing with tap water. This phenomenon was reported by Hass (1942). After removal from the formic acid, gross differences could be observed between the elastic residues of the copper-deficient and control pigs. The former were limp, their sides were collapsed and adherent to each other, and gross linear defects were seen separating the walls into layers. The loops of elastin residue from the controls maintained their oval shape and had a homogeneous appearance without gross defects. Both control and copper-deficient residues were white and shiny. The copper-deficient loops, before extraction, were thicker than the controls, but after extraction they were of approximately the same thickness. A comparison of dry weights before and after extraction is shown in Table 7. The percentage of elastin residue after formic acid extraction in the control was almost twice that in the copper-deficient aortas. The copper-deficient supplemented group fell within the same range as the deficient group without the supplement. These results indicate a greater proportion of formic acid soluble material in the deficient aortic loops.

It became important, therefore, to learn whether the aortic

Table 7.

Percentage of Insoluble Elastin Residue after Formic Acid Extraction

<u>Group</u>	<u>No. pigs</u>	<u>Residue in percentage of original weight</u>
I Control	12	44 \pm 5.7
II Copper-deficient	14	24 \pm 4.3
III Copper-deficient, supplemented	7	20 \pm 1.1

The mean values \pm one standard deviation are given.

elastin of the copper-deficient animals was reduced in amount or whether it had a lower resistance to formic acid. This could not be learned without determining elastin content by another independent method. However, it was possible to show that the curve of weight loss of the deficient aortas was like that of the controls. Therefore, the percentage of the residue after 36 hours extraction gives a valid comparison of the relative amounts of insoluble residue. The aortic rings from 6 control and 5 deficient pigs were washed, dried, and weighed after successive intervals of 36, 72, and 108 hours extraction. The percentages of residue at each time are shown in Table 8. It is evident that the loss of weight in the first 36 hours accounts for the difference between the copper-deficient and control aortas. The rates of loss between 36 and 108 hours are alike. These rates are slow but they are somewhat faster than that of the older, slaughterhouse pigs (Fig. 40).

During stretching there were qualitative differences between the groups in the manner of rupture. The control loops broke sharply and cleanly with no visible defect before the break. The deficient loops frayed progressively from the intimal aspect toward the periphery. This phenomenon was similar to that observed in the breaking pattern of whole fresh copper-deficient aorta and produced a similar aberration in the load-extension graphs. A comparison of typical control and copper-deficient breaking curves is shown in Fig. 42. The curves for the controls rose and fell sharply. The copper-deficient load-extension curves were not as steep, and at a lower tension the stress irregularly decayed as the different layers broke at different elongations, giving a ragged appearance to the curve. A composite graph of many of these

Table 8.

Rate of Weight Loss of Aorta in Formic Acid

<u>Time in formic acid (hours)</u>	<u>Residue in percentage of original weight</u>			<u>Ratio II/I</u>
	<u>I Control (6 pigs)</u>	<u>II Copper deficient (5 pigs)</u>		
36	46.7 (42.6-52.0)	23.9 (19.5-27.8)		0.51
72	39.9 (35.3-44.8)	21.0 (17.0-25.6)		0.52
108	32.2 (29.6-34.4)	17.6 (14.2-21.4)		0.54

Values given are the means with ranges in parentheses.

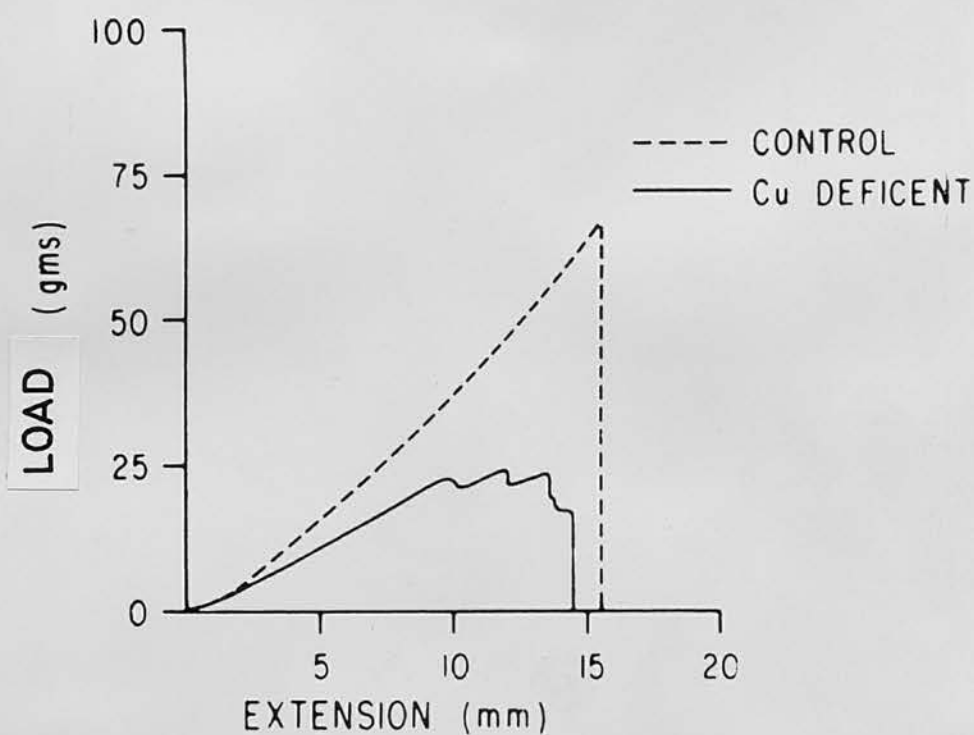


Fig. 42. Load developed during extension of aortic loops at constant rate to the breaking point. These are superimposed tracings of typical curves.

curves can be seen in Fig. 43. It is evident that the curves are monophasic, in contradistinction to the biphasic curves of stretched whole aorta. After making allowance for the differences in scale of the "load" ordinates, it is obvious that the load-extension curve of isolated aortic elastin corresponds with the 1st phase of the load-extension curve of whole fresh aorta. This supports the generally accepted attribution of the 1st phase of this curve to elastin.

Table 9 compares the tensile strengths of the control and the copper-deficient aortic residues. The tensile strength was calculated from the load-extension curves as $F_{\max}/2A$, where F_{\max} is the force in kilograms at the breaking point and A is the area calculated as a rectangle from the thickness and width of the cross-section at the narrowest point of the ring. An average was taken of 2 or 3 loops from each animal. The tensile strength of the control loops was about 3 times that of the deficient loops. There was no significant difference between the extensibilities of control and copper-deficient loops (Table 9).

A stretch modulus for a curve was again derived from the best tangent to the curve, using the formula $M = \frac{\Delta F/2A}{\Delta L/L}$ as shown in Fig. 44. The mean modulus of the controls was more than twice that of the deficient (Table 9).

Discussion

The morphologic changes in the aortas of copper-deficient swine and the stretch analysis of whole aorta pointed to a lesion of elastic tissue. The evidence from this experiment gives further support for this belief. There is a lower than normal

Table 2.

Mechanical Properties of Aortic Elastin

<u>Group</u>	<u>No. pigs</u>	<u>Tensile strength (kg/cm²)</u>	<u>Extensibility (% original length)</u>	<u>Elastic modulus (kg/cm²)</u>
Control	6	1.86 ± 0.85	131 ± 21.6	1.4 ± .43
Copper-deficient	9	0.57 ± 0.15	120 ± 17.1	.65 ± .16

Values given are the means ± one standard deviation.

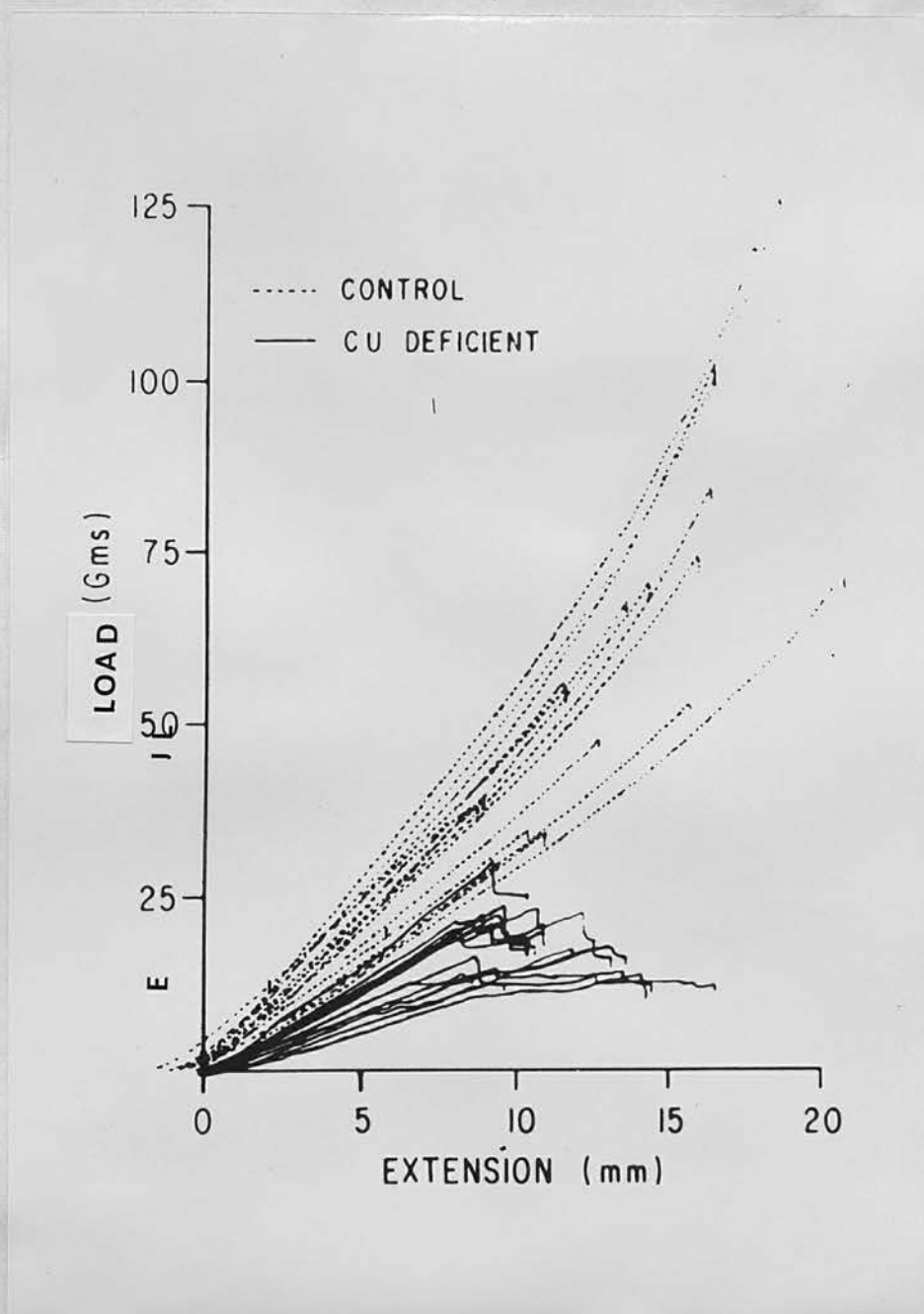


Fig. 43. Load-extension curves of elastin residue. The curves have been superimposed so that the tangents pass through the origin (see Fig. 44). The straight fall to the baseline after breaking has been omitted for clarity. Each curve represents a sample from a different pig.

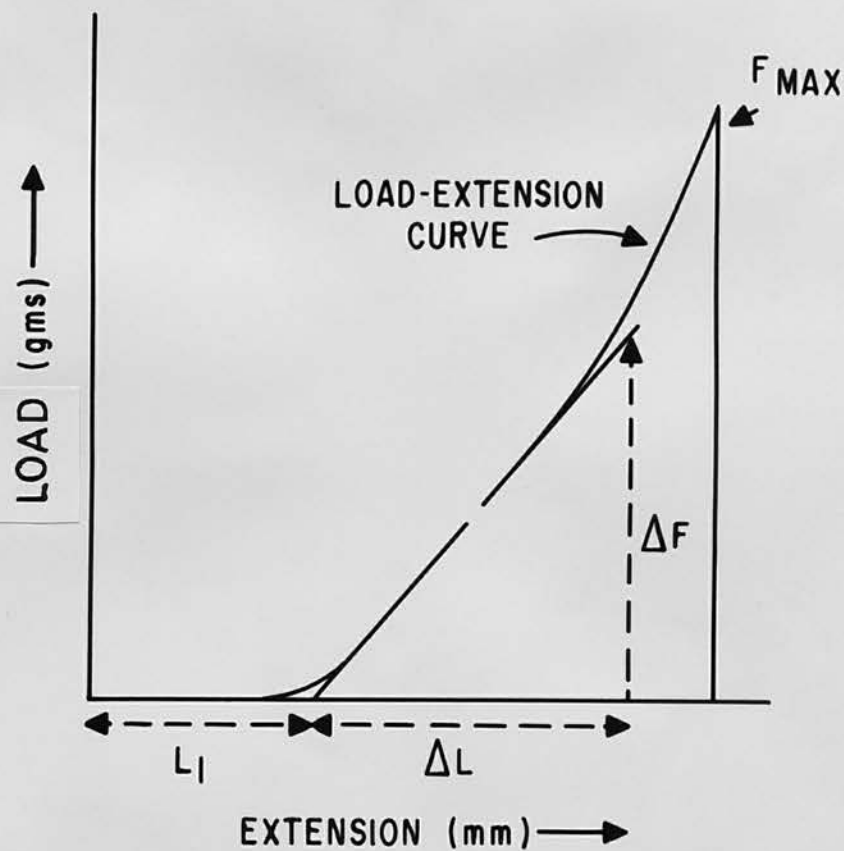


Fig. 44. Determination of elastic modulus. The calculations are explained in the text.

amount of insoluble elastin in copper-deficient aortas, reflecting either an impairment of its synthesis, a modification of its susceptibility to formic acid solubilization, or both of these. Recent work by Weissman et al. (1965) confirms the presence of a decreased elastin residue in the aortas of copper-deficient swine. Using complementary techniques, of autoclaving for an elastin residue and elastase digestion for elastin removal, they derived almost identical values from the two methods of 70.5% elastin in control aortas and 59% elastin in copper-deficient aortas. These values conflicted with those for elastin content by formic acid treatment, which they also measured, of 54% in the control and 33% in the copper-deficient aortas. These last figures are in keeping with elastin percentages found in this experiment (44% and 24% respectively), also using formic acid treatment. It was realised in the present experiment that formic acid does attack elastin and will eventually produce complete solubilization, but the important result of the study by Weissman et al. (1965) was that the elastin of copper-deficient pig aorta was very much more readily attacked than that of control aorta. It was calculated that 80% more elastin was dissolved by formic acid from the copper-deficient than from the control aortas. Moreover earlier work (Weissman, 1963) had shown that the elastin residue of aortas from copper-deficient swine, obtained by autoclaving, had a significantly lower content of proline, and also (Weissman, 1964) twice as much lysine, and significantly more aspartic acid and glutamic acid than controls. This suggested that copper deficiency had resulted in the formation of an altered elastin, which was more soluble in formic acid. Examination of the buffer soluble protein fraction from copper-

deficient aortas had revealed a proline:hydroxyproline ratio, characteristic of elastin (Weissman et al., 1963). This further suggested that a fraction of the elastin in these aortas was soluble and that there was either an aberration of synthesis or that the defective insoluble elastin underwent local lysis. Starcher et al. (1964) showed that after incorporation of valine-1-C¹⁴ into the aortic elastin of copper-deficient chicks, the radioactivity did not fall during a period of six days. This indicated that there was no increase in the rate of catabolism of elastin in the copper-deficient animal. However, the rate of incorporation of the radioactive valine was considerably retarded in the deficient chick during the first few days, suggesting an inhibition of synthesis of elastin in the deficient state.

The presence of defective elastin in the aortas of copper-deficient swine would account for the alteration in mechanical properties of the isolated elastin residue and also for changes in the 1st phase of the stretch response of whole untreated aorta. A possible mechanism to explain this will be dealt with later. However, the question remained - "Would the defect in elastin also account for the loss of tensile strength of whole intact copper-deficient swine aorta and the altered modulus of the 2nd phase of its stretch response?" It was considered that help in answering this problem might be gained by investigating the mechanical properties of an isolated aortic collagen residue.

Experiment 4.

The mechanical properties of isolated aortic collagen

One of the properties of elastin, which allows for the relative ease of isolating it from the other constituents of aorta, is its chemical inertness. Conversely, this makes the isolation of collagen, by chemical removal of elastin, an impossibility. Balo and Banga (1950) reported the existence of a pancreatic elastase which removed elastin from the aorta. At the time (1963), its specificity had not been seriously questioned and it was proposed therefore to use this method for the isolation of collagen from the aortas of copper-deficient and control pigs. It was also felt that should a defect in aortic collagen become apparent, similar changes in the collagen of other tissues, such as skin, might be expected, and accordingly skin was also tested.

I am indebted to D. T. Reay for carrying out the enzyme assay and to Dr. N. Weissman for the biochemical analysis.

Experimental

Fourteen animals from 2 litters of cross-bred swine were reared from the age of 4 days. All received iron and other minerals, but 7 were also given copper, serving as controls. Two of the copper-deficient animals died with haemopericardium at 95 and 105 days of age, and the other 12 animals were killed by exsanguination under anaesthesia. The five remaining copper-deficient animals were from 61 to 84 days old, and the age range of the seven control animals was 61 to 110 days.

Segments from the descending thoracic aorta were cut free-hand

into rings of as near 3 mm width as possible, avoiding branches, and stored in individual tightly capped vials in an ice-bath until stretched. This period never exceeded 24 hours. After stripping loose adventitia, the rings were weighed, and mean values for width and thickness measured under a calibrated stereomicroscope. Successive rings were set aside for immediate measurement of mechanical properties, and for incubation with buffered elastase and with buffer alone. Each aorta provided five rings for each of these procedures.

An aqueous crystalline suspension of porcine pancreatic elastase (Worthington Biochemical Corporation, Freehold, New Jersey) was dialyzed three times at 2°C against 0.05 M Tris buffer (pH 8.8) for 1 hour. After diluting 1 part in 10 with the same buffer, the enzyme preparation was assayed photometrically with an elastin-orcein substrate as described by Sachar et al. (1955). The specific activity (Worthington Biochemical Corporation, 1964) varied between 25 and 35 units of elastase per mg. In order to assess the optimum time for total digestion of elastin, a preliminary experiment was undertaken with aortic rings from farm-bred pigs of a size equivalent to that of the experimental animals. The rings were incubated in a shaking bath with an elastase suspension at 37°C for periods of 1, 2, 6, 12, 18, and 24 hours. The rings were examined histologically for residual elastin, and the supernatant was analysed for soluble protein by the biuret reaction. Biochemical analyses indicated that 12 hours incubation at 37°C was sufficient for the removal of all medial elastin from an aortic ring of average weight 150 mg (Weissman et al., 1965).



The experimental aortic rings were accordingly incubated for 12 hours at 37°C in both the buffered elastase preparation and buffer alone. At the end of this time the rings were repeatedly washed in buffer, and if not tested immediately, were stored in a cold room for up to 8 hours. Load-extension curves were prepared as already described. The broken rings were washed with distilled water, dried in vacuo, and weighed.

In order to quantitate the elastic behaviour a value for cross-sectional area is essential. I have used three sets of values. The first was a product of the direct measurements of ring width and thickness described above. In order to approximate the collagen component a second area was calculated from this based on the percentage of collagen in dry fat-free aorta: 17.3 per cent for control and 18.0 per cent for copper-deficient swine (Weissman et al., 1963). Although these values for cross-sectional area could still be applied to the rings incubated in buffer alone, they bore little relationship to the rings digested with elastase. The third method was based on estimated cross-sectional areas of elastase-digested rings with the use of a densitometric method. Nine copper-deficient and 12 control, elastase-digested, aortic rings were thoroughly washed in distilled water, pressed dry between filter papers, using as near possible constant pressure, and weighed. Approximate density measurements were calculated by flotation in mixtures of xylol and brombenzene. There was no difference in density between copper-deficient and control tissues, which measured 1.03 ± 0.01 . The rings were then dried in vacuo and reweighed. The ratios of wet weight to dry weight were 8.8

± 1.5 for copper-deficient and 17.2 ± 2.7 for control tissues.

With these factors, hypothetical wet weights were calculated on the stretched experimental rings, the dry weights of which were known. To have attempted direct measurements of wet weights on these rings would have necessitated removal of excess moisture with filter papers which would have rendered them useless for stretching.

When the wet weight and density were known, the wet volume and

conditions as described above for aorta for 24 hours, and the remaining seven loops in buffer alone for the same time. These loops were then also stretched to breaking point.

Results

Aortic Collagen

The elastase-digested aortic rings presented an expanded, loose-textured, translucent, myxoid appearance, making accurate width and thickness measurements impossible (Fig. 45). Histologic sections stained by the Verhoeff-van Gieson method showed a complete absence of elastin. Collagen fibres were tinctorially normal but the cells of the vessel wall showed advanced lysis (Fig. 46). Those segments incubated in buffer alone were grossly unchanged, and microscopically showed moderate cell autolysis only.

Figures 47 and 48 show typical stress-strain curves on the copper-deficient and control aortic rings, respectively. In each figure, curve 1 represents the buffer incubated and curve 2 the elastase-digested ring. The buffer-incubated rings exhibited the typical two-phase curve of aortic tissue. After elastase digestion there was virtual elimination of the first phase, leaving a curve qualitatively similar to the second phase of intact aorta. Quantitatively this phase was markedly altered. Ultimate tensile strengths, as breaking strength per unit cross-sectional area, are recorded in Table 10. Stretch moduli, derived from the best tangent to the elongation curve, as described in Experiment 2, are shown in Table 11. The ultimate tensile strengths and stretch moduli were sharply reduced in both the control and the copper-deficient specimens, by all three methods of cross-sectional area

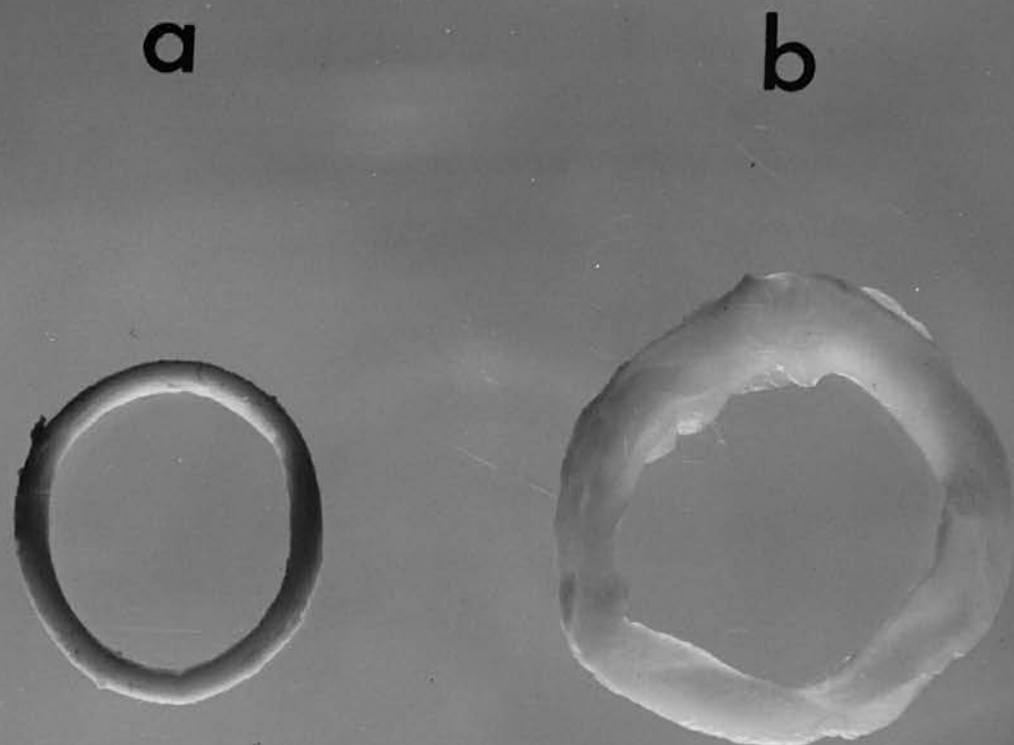


Fig. 45. Aortic rings after incubation in buffer only (a) and elastase (b) illustrating the expansion and translucency produced by elastase.

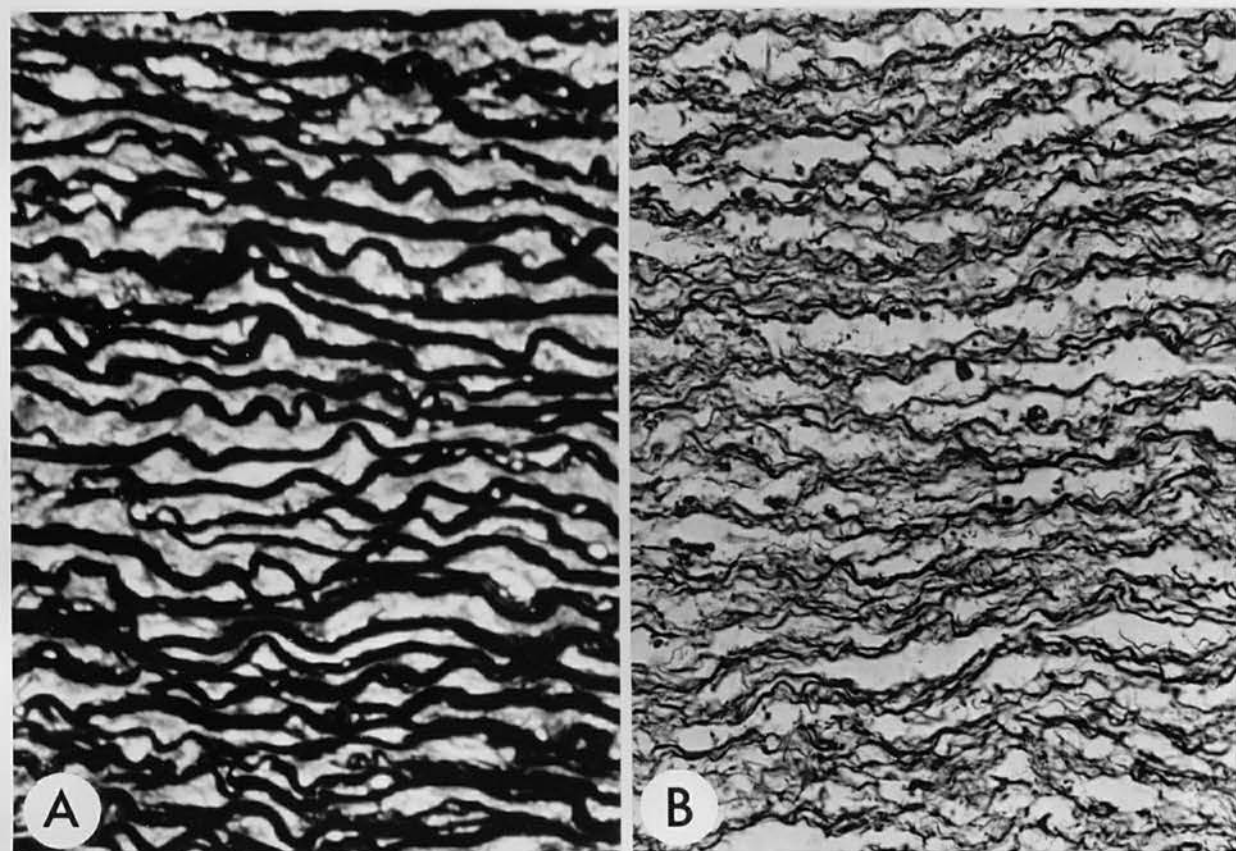


Fig. 46. Aorta from control diet swine after incubation in buffer only (A) showing normal elastin laminae, and elastase (B), showing the absence of elastin but leaving tinctorially normal collagen. Verhoeff-van Gieson; x 100.

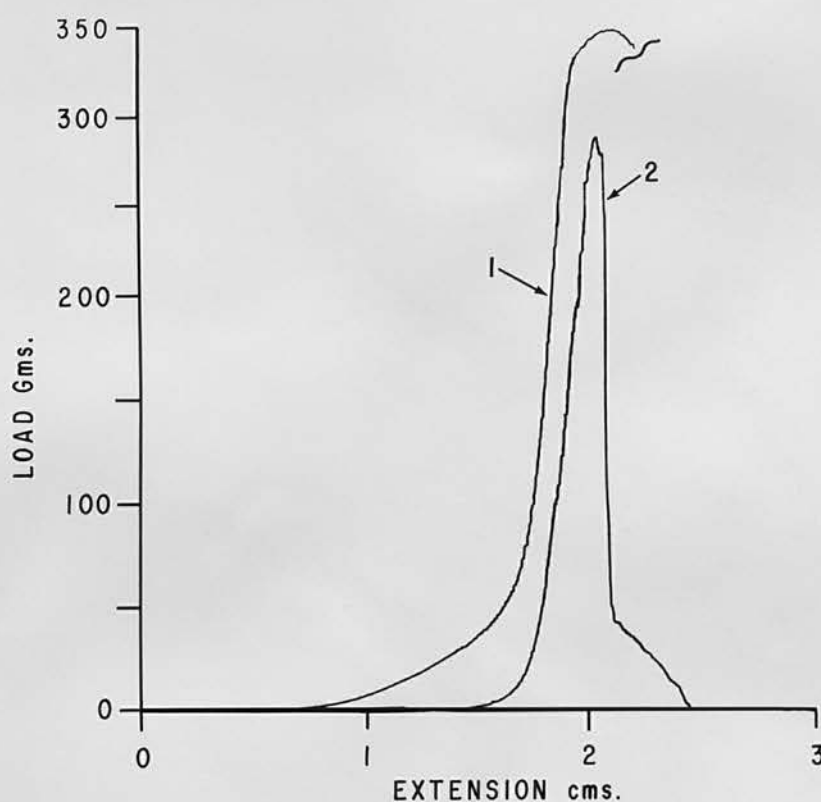


Fig. 47. Load-extension diagrams of aortic loops from a copper-deficient pig, after incubation with buffer only (curve 1), and elastase (curve 2). Both were stretched to breaking point. The first of the two extension phases seen in curve 1 is virtually absent from curve 2, which also has a lower breaking strength.

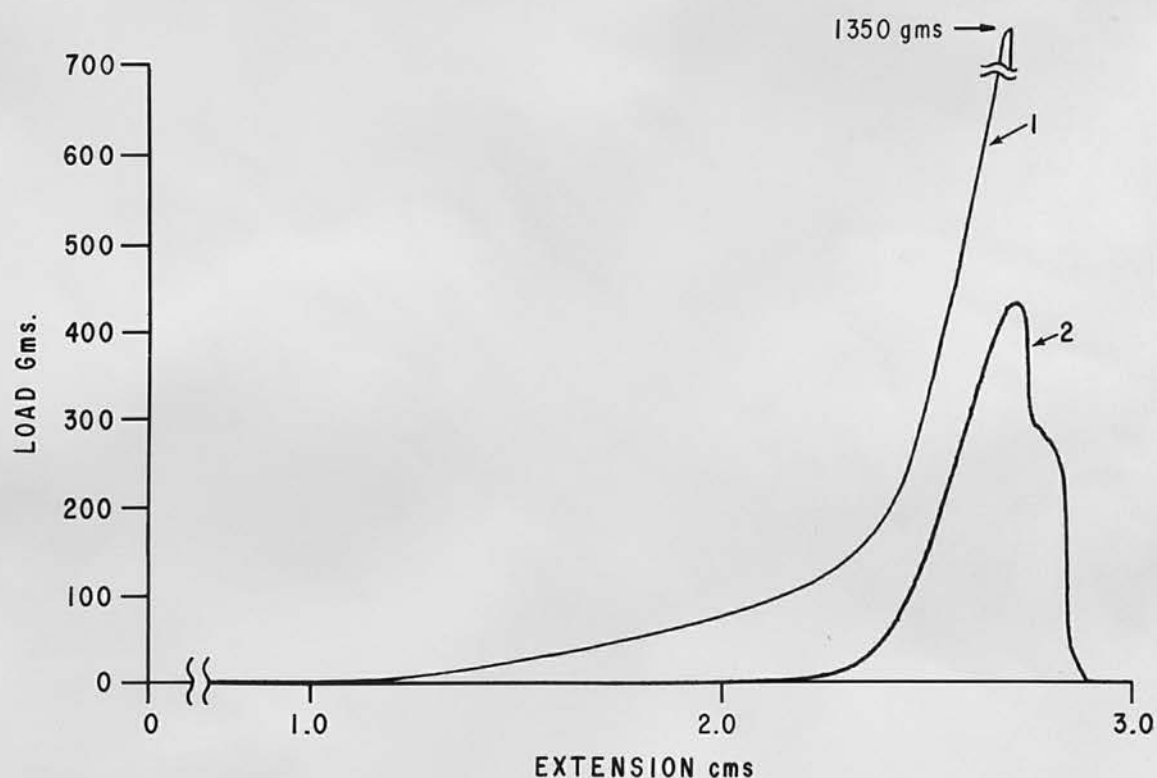


Fig. 48. Load-extension diagrams of aortic loops from a control pig, after incubation with buffer only (curve 1) and elastase (curve 2). The same changes as noted in Figure 47 are present.

Table 10.

*
Ultimate Strength of Aorta as Affected by Elastase

<u>Group</u>	<u>Pigs</u>	<u>Rings</u>	<u>Buffer solution only</u>		<u>Elastase-digested</u>		
			A	B	A	B	C
		no.	kg/sq.cm		kg/sq.cm		
Control	7	63	16.8 ± 4.4	97.8 ± 25.6	4.2 ± 1.2	24.5 ± 6.8	5.9 ± 2.0
Copper-deficient	7	61	6.3 ± 1.9	35.7 ± 10.9	1.8 ± 0.9	10.3 ± 5.1	3.1 ± 1.5

* Column A, cross-sectional areas calculated by direct measurement of width and thickness of original untreated aortic rings; column B, calculations as in column A with a correction for estimated collagen content; column C, cross-sectional areas estimated by densitometric method.

Table 11.

Stretch Modulus of Aortic Collagen as Affected by Elastase *

<u>Group</u>	<u>Pigs</u>	<u>Rings</u>	<u>Buffer solution only</u>		<u>Elastase digested</u>		
			A	B	A	B	C
			kg/sq.cm/100% elongation		kg/sq.cm/100% elongation		
Control	7	63	111.5 ± 35.5	620 ± 85	36.5 ± 13.6	205 ± 76	42.7 ± 18.8
Copper-deficient	7	61	41.2 ± 14.1	208 ± 73	13.0 ± 6.8	62 ± 40	19.6 ± 11.9

* See Table 10 for explanation of columns.

measurement. The buffer control values agree substantially with those for untreated aortic rings reported in Experiment 2.

Skin Collagen

A breaking stretch curve of a typical fresh skin loop is shown in Figure 49, and resembles those reported for collagen from other tissues (Partington and Wood, 1963; Rigby et al., 1959). After the initial "toe," the curve is approximately straight before finally yielding and breaking. Stretch moduli were calculated from the straight part of the curve, and ultimate tensile strengths as breaking load per unit cross-sectional area. The results are shown in Table 12. There is no significant difference for either set of values between control and copper-deficient specimens.

When loops of skin were incubated with elastase for 24 hours, stainable elastin was completely eliminated. Other observations included the sloughing of the epidermis as an intact structure and the development of a sulphurous odour. None of the buffer-incubated specimens behaved similarly. Specific staining for bacteria failed to reveal any evidence of such proliferation. The results of mechanical stretching are shown in Table 13. The low values for mean ultimate strength of fresh untreated specimens in comparison with those of the earlier experiment (Table 12) may be explained on the age difference of the two groups (Rollhäuser, 1950). Although soaking in buffer alone at 37°C for 24 hours has reduced the tensile strength by more than half, the action of elastase has produced relatively little further weakening. In all three groups the shape of the load-strain curve was the same as that in Figure 49.

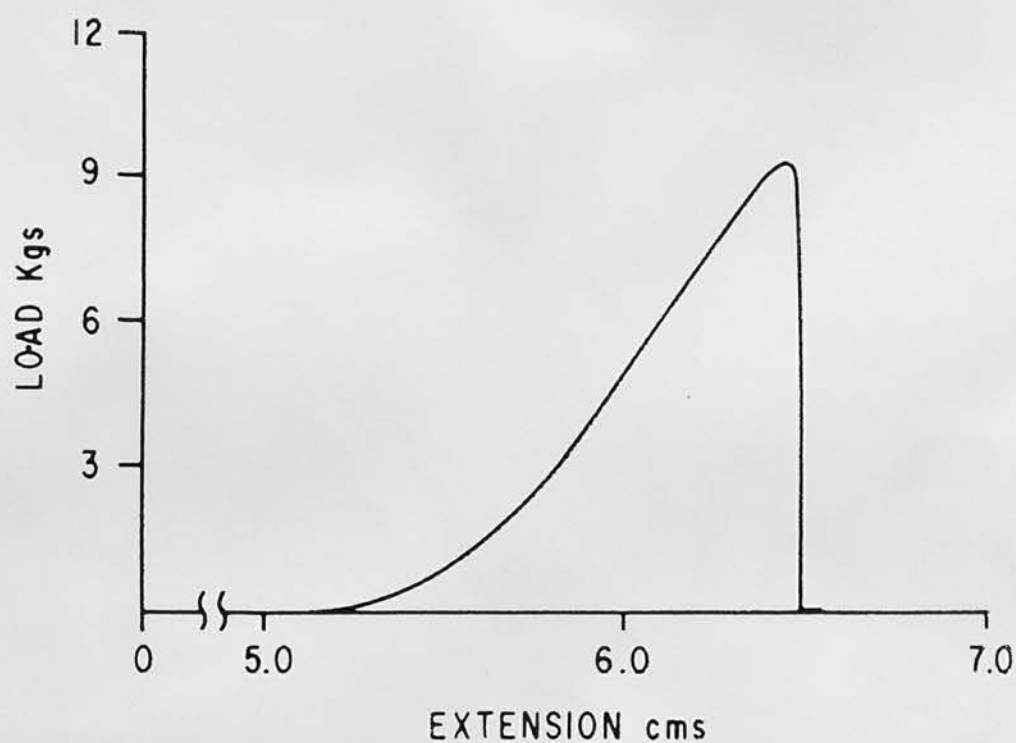


Fig. 49. Load-extension diagram of a fresh skin loop from a control pig.

Table 12.

Mechanical Properties of Skin Loops

<u>Group</u>	<u>Pigs</u>	<u>Skin loops</u>	<u>Ultimate strength</u>	<u>Stretch modulus</u>
		no.	kg/sq.cm	kg/sq.cm/100% elongation
Control	2	6	104 \pm 45	577 \pm 195
Copper- deficient	3	9	92 \pm 21	518 \pm 214

Table 13.

Ultimate Strength of Skin as Affected by Elastase

<u>Group</u>	<u>Fresh skin</u> <u>loops</u>	<u>kg/sq.cm</u>	<u>Buffer-only</u> <u>incubated</u> <u>skin loops</u>	<u>kg/sq.cm</u>	<u>Elastase-</u> <u>digested skin</u> <u>loops</u>	<u>kg/sq.cm</u>
Control		50.4 (3)		14.7 (1)		9.5 (2)
Copper- deficient		41.3 (4)		17.1 (2)		16.8 (2)
Copper-deficient treated		34.1 (6)		15.7 (4)		11.6 (2)

Figures in parentheses denote the number of skin loops tested.

Discussion

In his review on the relation of structure to function of the tissues of the wall of blood vessels, Burton (1954) compares vascular collagen and elastin to a simple "in parallel" arrangement of string and rubber, and other authors have followed his example (Bader, 1963; Bergel, 1961; Roach and Burton, 1957). Wolinsky and Glagov (1964) have advocated comparison of aorta to a "two-phase material" such as Fibreglass, in which glass fibres (collagen) are embedded in a plastic matrix (elastin). The analogy is not strict since collagen is not embedded in elastin, but this hypothesis does serve to stress the possibility of an actual bond between collagen and elastin. As recent an authoritative work as that of Remington (1963) draws an analogy to a rubber tube wrapped with a fibrous jacket. Such a jacket may be considered to exist in the tunica adventitia, but a considerable part of the aortic collagen lies in the media in intimate association with elastin and muscle cells.

After elastase digestion of aorta, there were decreases in ultimate tensile strength and stretch modulus for both control and copper-deficient specimens. The extent of the decrease in ultimate strength was considerably in excess of the decrease expected from the contribution of elastin itself in a simple parallel arrangement. The comparison is shown in Table 14.

These results appear to contradict those of Roach and Burton (1957), who removed the elastin of human external iliac arteries with crude trypsin. Although they did not load to breaking point, they implied that the collagen residue had a comparable modulus to that of the second phase of the intact vessel.

Table 14.

Comparison of Decrease in Ultimate Strength of Aorta after Elastase Digestion and of
Ultimate Strength of Aortic Elastin Residue *

Group	Decrease in ultimate strength after digestion		Ultimate strength of aortic elastin [‡]
	A	B	
Control	12.6	73.3	1.86
Copper-deficient	4.5	25.4	0.57

* Column A, values derived on the basis of cross-sectional area by direct measurement of width and thickness of original untreated aortic rings; column B, calculations as in column A with a correction for estimated collagen content.

[‡] Data from Experiment 3.

The present results would indicate that some component removed by elastase contributes substantially to the second phase of the aortic stress-strain curve. The enzyme preparation used had negligible proteolytic activity when tested against a commercial bovine collagen preparation in the same buffer and at the same pH (Weissman et al., 1965). It appeared unlikely, therefore, that collagen degradation was responsible for these effects. Assuming that the only difference between buffer and elastase-digested specimens was the absence of elastin in the latter, it would appear that the presence of elastin is essential to the integrity of the collagen framework, suggesting some form of mutual bonding.

The results on skin elasticity fall within the same range as measurements of tensile strength on human skin as recorded by Rollhäuser (1950) of 25 to 160 kg per sq.cm, depending on age, and on rat leg skin by Fry et al. (1964) of 150 to 550 kg per sq.cm, also depending on age. The absence of significant difference in quantitated mechanical properties between control and copper-deficient skin would indicate that a change in collagen, which is practically the sole source of cutaneous strength, is not a feature of copper-deficiency. In support of this contention, Weissman et al. (1963) found no difference in the quantity or solubility of collagen in the copper-deficient swine aorta, or in the amino acid analyses of the collagen. The extremely low hydroxyproline content of the saline soluble extract was essentially the same in the deficient and control pigs. Moreover, elastase did not produce a striking alteration in mechanical properties of skin such as it did in the much higher elastin containing aorta.

It is of interest that the tensile strength of control aorta (97.8 kg per sq.cm), after adjustment for the concentration of collagen present, is of the same order as that of control skin (104 kg per sq.cm) in this experiment, whereas the tensile strength of tendons varies from 500 to more than 1,000 kg per sq.cm (Elliott, 1965). This almost certainly reflects the pattern or orientation of collagen fibres within the tissue, from parallel in tendon, to networks in skin and aorta. In fact, in skin and aorta, tissue breakage is most probably not a function of ruptured collagen fibres so much as slippage between adjacent fibrils. One could conclude, therefore, that in copper-deficiency the alteration in ultimate tensile strength and the stretch modulus of the second phase of an aortic stress-strain curve, theoretically a function of collagen, is the result of a defect in elastin, and that elastin contributes to the mechanical properties of the collagenous component by binding its fibrils together.

Subsequent to the completion of this experiment, Drake et al. (1966) reported on the action of elastase and other proteolytic enzymes on tropocollagen and insoluble collagen. They concluded that elastase attacked the extra-helix peptide appendages, the so-called telopeptides, through which most of the intra- and inter-molecular cross-links of collagen occur, without changing its main structural features. If this is correct, it could explain the decrease in aortic tensile strength after elastase treatment, but not the absence of a similar effect on skin. This might still, of course, reflect the nature and magnitude of intermolecular linkages in skin collagen as compared with the aortic collagen-elastin

framework. At this point the problem rests, for there is apparently no certain way of removing elastin from a tissue without at the same time affecting the collagen.

Experiment 5.

The mucopolysaccharide ground substance and the mechanical properties of the aorta

The presence, in abnormal amounts, of an amorphous ground substance in the aortas of pig suffering from advanced copper deficiency has been described in Experiment 1. Part of this material was strongly metachromatic with toluidine blue, indicating its mucopolysaccharide nature and was defined both quantitatively and qualitatively in a study made in association with Linker (1964). The copper-deficient aortas contained about 3 times as much mucopolysaccharide as the controls. This increase was shown to be due to a rise in the content of chondroitin sulphate B and chondroitin sulphate A or C, or both A and C, while the heparitin sulphate level remained essentially unchanged.

Despite the ubiquity of "ground substance" and the constant relationship between collagen and elastin and mucopolysaccharides, such reviewers as Burton (1954) and Remington (1963) failed to mention mucopolysaccharide in connection with aortic mechanical properties. In studies on the part played by mucopolysaccharide in the mechanical behaviour of elastic tissue from ox ligamentum nuchae, Wood (1954) concluded that hyaluronidase treatment produced marked weakening of the native tissue, but had little effect on the elastin residue left after boiling with dilute acetic acid. In further studies on the effect of enzymatic removal of mucopolysaccharides from rat-tail tendon, an almost pure collagenous tissue, Partington and Wood (1963) reported that chondroitin sulphate A and C and hyaluronic acid were not important in stabilizing the collagen

fibres. Several of their hyaluronidase preparations, however, did produce a significant weakening which they attributed to proteolytic enzyme contamination. Despite this, incubation of tendon with crystalline trypsin produced less effect than the hyaluronidase preparations and was not thereafter studied in detail. Harkness and Harkness (1959), on the other hand, reported no effect of hyaluronidase, but a pronounced effect of trypsin on the mechanical properties of both pregnant rat cervix and the skin of new-born rats.

The disparity of these results, the widely-held concept of ground substance mucopolysaccharide as a tissue glue, and the known alteration of this component in the aortas of copper-deficient pigs, suggested the next experiment in this series. Unfortunately, it had to be a "mirror image" of the two preceding experiments, insofar as a "residue" of aortic mucopolysaccharide was useless for the measurement of mechanical properties. Consequently the residues following removal of the mucopolysaccharide content of copper-deficient and control aortas were tested in the usual way. All previous experiments involving the removal of mucopolysaccharide from aortas for purposes of measuring mechanical properties had used testicular hyaluronidase. This enzyme is effective against hyaluronic acid and chondroitin sulphates A and C, but not against chondroitin sulphate B and heparitin sulphate. It was therefore decided to use in addition to hyaluronidase, a Flavobacterial enzyme known to degrade all the aortic mucopolysaccharides. The enzymes were supplied by Dr. Alfred Linker, to whom I am also indebted for the biochemical analyses.

Experimental

A total of nineteen cross-bred pigs from several litters, including one of miniature breed, were raised from four to five days of age on the copper-deficient milk diet, supplemented with iron and other minerals. Six of these animals also received copper and served as controls. Three of the copper-deficient animals died at ages of 104 to 109 days, all with rupture of the ascending aorta. The remainder of this group was killed by exsanguination under anaesthesia at 74 to 109 days of age. The control animals were similarly killed at ages of 71 to 106 days. At autopsy, segments of approximately 3 cm length were removed from the middle sector of the descending thoracic aorta and stored in tightly capped vials at 4°C until used. After stripping loose adventitia, each segment was manually sliced into uniform rings from 1 to 2 mm in width. The twelve best rings from each segment were divided into three or four groups, according to the experiment.

Experiment A

The rings were divided into three groups of four and treated as follows:-

Group 1. These rings were immediately tested for mechanical properties, after making direct measurements of average ring widths and thicknesses under a calibrated stereomicroscope. Each ring was subjected to two conditioning stretches, short of the yield point, before being stretched to breaking point. During this process, which lasted approximately 30 minutes for each specimen, the rings were immersed in physiological saline.

Group 2. The rings were weighed and transferred to vials

containing 1.0 ml of 0.025 M phosphate buffer, pH 7.0

Group 3. The rings were weighed and placed in vials containing 1.0 ml of 0.025 M phosphate buffer, pH 7.0, in which was dissolved 2 mg of a Flavobacterial mucopolysaccharidase, prepared as described by Linker and Hovingh (1965).

The vials of both Groups 2 and 3 were incubated at 25°C for 16 hours, following which the rings were washed several times in distilled water and stored in closed vials at 4°C. Measurement of average width and thickness, and stretching were performed within 12 hours, in the same way as the untreated rings of Group 1. The incubation solutions from Groups 2 and 3 were analyzed for uronic acids by the carbazole method (Dische, 1947).

Experiment B

The rings were again divided into three groups of four and, after weighing, incubated for 16 hours in the following solutions.

Group 1. 1.0 ml of 0.1 M acetate buffer, pH 5.0 at 37°C.

Group 2. 1.0 ml of 0.1 M acetate buffer, pH 5.0, containing 2 mg of testicular hyaluronidase (California Biochemical Research), 300 USPU/mg at 37°C.

Group 3. 1.0 ml of 0.025 M phosphate buffer, pH 7.0, containing 2 mg of Flavobacterial mucopolysaccharidase, at 25°C.

Experiment C

The rings were divided into four groups of three, which were incubated in 1.0 ml of the following media:

Group 1. 0.025 M phosphate buffer pH 7.0, at 25°C.

Group 2. A combination of testicular hyaluronidase and Flavobacterial mucopolysaccharidase in 0.025 M phosphate buffer pH 7.0, at 25°C.

Group 3. Crystalline trypsin, 0.5 mg per ml (kindly supplied by Dr. Sherman R. Dickman, Department of Biochemistry, University of Utah) buffered in 0.025 M phosphate, pH 7.0, at 37°C.

Group 4. Crystalline trypsin as in Group 3 above, followed after washing, by combined testicular hyaluronidase and Flavobacterial mucopolysaccharidase as in Group 2 above.

The aortic rings from Experiments B and C were subsequently stretched to breaking and the supernatants analyzed for uronic acids, as described for Experiment A. Immediately after rupture all rings were placed in Newcomer's fixative, embedded in paraffin and sectioned following the method described by Saunders (1964). Sections were stained with alcian blue at pH 2.5

Results

The mean values of ultimate tensile strength, calculated as breaking force per unit cross-sectional area, are presented for Experiments A, B and C in Tables 15, 16 and 17 respectively. Values for untreated rings fell within the ranges previously described. The variation in aortic strength of the copper-deficient group reflected the extent of the deficiency in individual animals. The stress-strain curves following incubation in buffer and mucopolysaccharidase are shown as A and E respectively in Figure 50. They have the normal biphasic form of stretched untreated aorta. The ultimate tensile strength of aorta appears

Table 15.

Effect on aortic tensile strength of Flavobacterial mucopolysaccharidase

<u>PIG NO.</u>	<u>STATUS</u>	<u>Ultimate tensile strength of aorta</u> <u>kg/sq.cm</u>		
		<u>Untreated</u>	<u>Buffer treated</u>	<u>Flavo mucopolysaccharidase</u> <u>treated</u>
1814	Control	23.5	22.4	25.6
1815	"	28.4	28.9	27.4
1799	Copper-deficient	14.1	11.8	12.1
1840	"	5.9	6.3	7.9
1843	"	2.0	2.1	2.5
1798	"	6.9	6.7	6.7
M32	"	9.7	11.7	13.3
1820	"	5.9	8.8	9.3
1842	"	9.3	8.2	10.0

Table 16.

Comparison of effects on Aortic Tensile strength of testicular hyaluronidase and

Flavobacterial mucopolysaccharidase

PIG NO.	STATUS	Ultimate tensile strength of aorta kg/sq.cm		
		<u>Buffer</u>	<u>Testicular Hyaluronidase</u>	<u>Flavo mucopolysaccharidase treated</u>
M39	Control	16.5	19.0	15.3
M38	Copper-deficient	0.9	1.8	1.5
M41	"	2.1	3.3	2.1
66	"	7.7	8.6	10.3

Table 17.

Comparison of effects on aortic tensile strength of mucopolysaccharidases and trypsin

FIG NO.	STATUS	Ultimate tensile strength of aorta kg/sq.cm			
		Buffer	Testicular hyal- uronidase + Flavo- bacterial muco- polysaccharidase	Trypsin	Trypsin + combined muco- polysaccharidases
54	Control	23.0	18.0	7.0	7.5
55	"	12.0	18.3	6.6	6.9
56	"	19.0	18.0	3.9	3.7
58	Copper-deficient	4.8	5.6	1.0	0.94
60	"	6.9	7.4	3.6	3.5
62	"	13.3	13.3	7.2	4.9

to be unaffected by incubation in either buffer alone or the two mucopolysaccharidases, whether used singly or in combination. There was no difference in behaviour between copper-deficient and control tissues.

Only small amounts of mucopolysaccharide, as measured by the uronic acid reaction, were extracted from the aortic rings following incubation. Although slightly more polysaccharide appeared in the supernatant of the enzyme-treated rings than in the buffer controls, the difference was not sufficient to be significant.

Those aortic rings treated with trypsin, on the other hand, showed a two- to five-fold reduction in aortic tensile strength. A typical stress-strain curve is shown as G in Figure 50. The curve remains biphasic but shows a diminution of slope in both phases, in addition to the low breaking stress. During the preliminary reversed conditioning stretches, the trypsin-treated ring, H in Figure 51, showed greater extension under the same stress than the buffer-treated ring A, or the mucopolysaccharidase-treated ring D. The hysteresis loop also appeared to have a diminished area. The supernatant following trypsin incubation contained relatively more mucopolysaccharide, of the order 0.1 mg per 100 mg of aortic ring (wet weight). The subsequent incubation of a trypsinized ring in mucopolysaccharidase produced no further decrease in tensile strength and did not liberate further significant quantities of mucopolysaccharide.

Examination of the stained rings revealed no significant difference in the amount of alcian blue positive material between untreated and buffer treated specimens. A much higher quantity of this material was present in the aortic rings from copper-

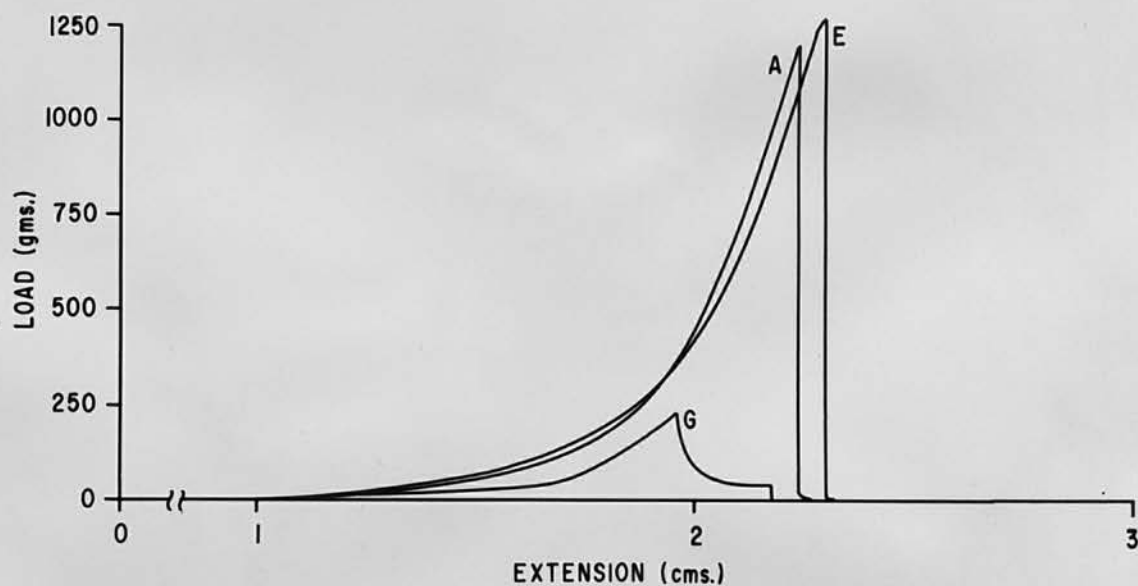


Fig. 50. Load-extension curves of aortic rings, A - after incubation in buffer only, E - following incubation in combined testicular hyaluronidase and Flavobacterial mucopolysaccharidase, and G - after incubation in trypsin.

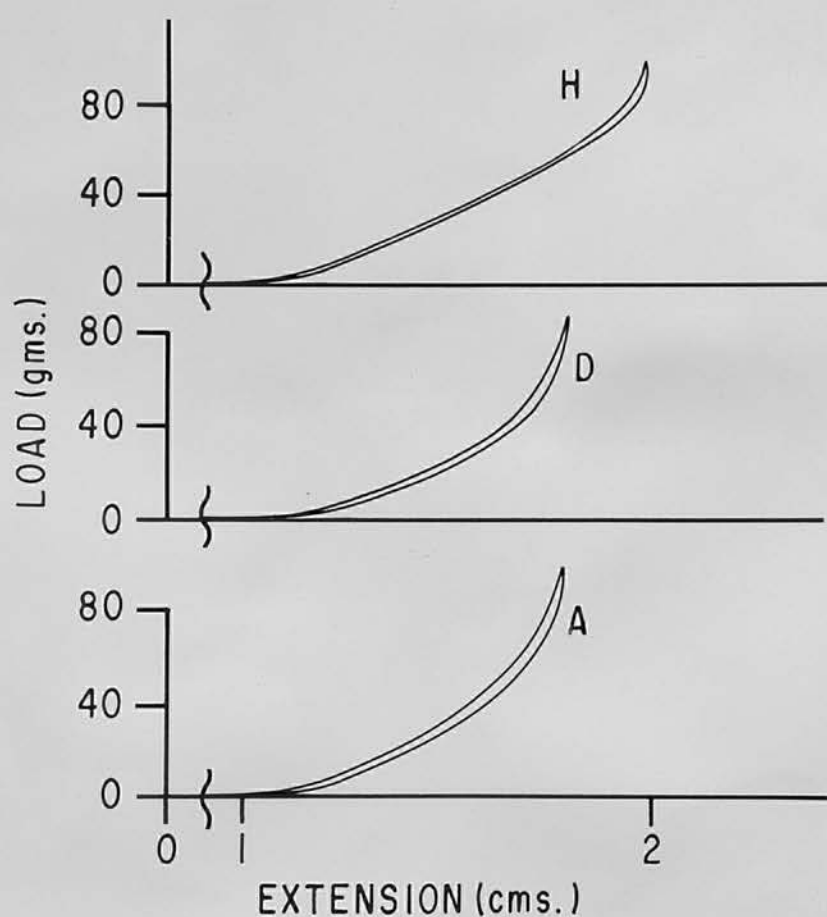


Fig. 51. Reversed load-extension (hysteresis) curves of aortic rings, A - after incubation in buffer only, D - following treatment with combined testicular hyaluronidase and Flavobacterial mucopolysaccharidase, and H - after incubation in trypsin.

deficient animals, as noticed previously. Those rings incubated with the Flavobacterial enzyme were completely devoid of alcian positive substance while those incubated with testicular hyaluronidase showed a significant reduction. The trypsinized rings showed little if any reduction of alcian positive material. There was no morphological evidence of bacterial proliferation during the experiment.

Discussion

Acid mucopolysaccharides readily bind to proteins, including collagen (Einbinder and Schubert, 1951). The mechanism of collagen binding was suggested by Jackson (1954) as 40% salt-like and 60% hydrogen bonding. In a preliminary paper (Jackson, 1953), he found that removal of chondroitin sulphuric acid from rat-tail tendon by testicular hyaluronidase resulted in increased fibre swelling, increased fibre solubility in dilute acetic acid and a reduction in shrinkage temperature. He concluded that chondroitin sulphuric acid was concerned with collagen inter-chain linkages. However, his enzyme preparation was crude and may have been also proteolytic. Wood (1960) studied the effects of mucopolysaccharides on collagen fibril formation from solution and concluded that chondroitin sulphates A and C accelerated formation, while chondroitin sulphate B and hyaluronic acid had no effect. In view of the very small amount of chondroitin sulphate incorporated in the precipitate, he postulated that mucopolysaccharides affect nucleation of fibrils. Using a synthetic mixture of collagen fibres, hyaluronic acid and water, Fessler (1960)

produced a meshwork of fibres by warming to 37°C. Centrifugal compression resulted in a pellet containing a higher water content than those formed in the absence of hyaluronic acid. He concluded that although the action of hyaluronic acid was most probably a mechanical one during centrifugation of the gel, it may also have influenced secondarily the formation of the collagen fibre meshwork. More recently Mathews (1964) has demonstrated electrophoretically the formation of a complex between pure acid mucopolysaccharide and stable soluble collagen, and concludes that electrostatic factors are of major importance. He proposed a model consisting of basic units of chondroitin sulphate-protein molecules, the chondroitin sulphate moieties of which are aligned in parallel with and electrostatically bound to collagen fibrils. Other work by Di Salvo and Schubert (1966), using cartilage protein polysaccharide precipitation of collagen from solution, led to their suggestion of a model involving entanglement between insoluble collagen fibrils and the relatively stiff chondroitin sulphate chains of branched protein-polysaccharide.

Most of this work has revolved around the formation of a collagen meshwork, and the question may be asked - "Does mucopolysaccharide affect the stability of established connective tissue?" The earlier report by Wood (1954) suggested that removal of chondroitin sulphates A and C and hyaluronic acid by testicular hyaluronidase weakened ox ligamentum nuchae, and three out of four similar enzyme preparations produced the same effect on rat-tail tendon (Partington and Wood, 1963). The fourth preparation did not weaken this tissue, leading to a conclusion that these mucopolysaccharides are not essential for the maintenance of the mechanical properties of connective tissue.

polysaccharides were not important in stabilizing the tissue, and that the other enzymes contained proteolytic enzyme impurities. The contention that the proteolytic action of these enzymes is directed only against a non-collagenous protein of the matrix is probably invalidated by the recent work of Drake et al. (1966). They showed the trypsin and other proteases break cross links between the α -chains of collagen both intra- and inter-molecularly, without affecting the α -chains themselves. This action could account for the severe weakening produced by trypsin in the aortic rings in this study and in the trypsinized tissues as reported by Harkness and Harkness (1959). Nevertheless, the fact that trypsin may attack the protein of the ground substance is indicated in this work by the higher levels of uronic acid detected in the medium following trypsin as compared with mucopolysaccharidase incubation. Despite this, alcian blue positive material remained in the tissue, a finding reported also by Benditt and French (1953) in cartilage exposed to trypsin, by Pepler and Brandt (1954) in both aorta and cartilage, and by Churchill et al. (1955) in control and lathyrctic rat aortas. Other proteases apparently may abolish metachromasia (Manley, 1964). This result was somewhat paradoxical. Mucopolysaccharidases reduced or abolished alcian blue staining of the aorta, but liberated little mucopolysaccharide into the incubation fluid; whilst trypsin had little effect on alcian blue staining, and freed comparatively more of the mucopolysaccharide into solution. If the mucopolysaccharidase failed to penetrate the aorta, despite their reputation as "spreading factors", - how was the loss of alcian blue positivity so uniform? When the ability

to take up alcian blue rests with the mucopolysaccharide moiety of its complex with protein, - how can it come into solution in relatively large amounts but still stain in the tissue? Assuming that trypsin liberates mucopolysaccharide by degrading the protein moiety of the complex, and at the same time drastically reduces tensile strength, may not this protein be important in holding the tissue together? Is the loss of tensile strength the result of a partial collagenolytic activity of trypsin? These questions were not answered in this experiment, which unfortunately seemed to raise more questions than it answered. The importance of the protein moiety of the ground substance in producing connective tissue stability will remain in doubt until some way of degrading it without simultaneously affecting collagen or elastin is found.

Milch (1965) suggested that mucopolysaccharides may be capable of acting as "plasticizers" rather than "adhesives" for fibrous proteins. He postulated a model for any native connective tissue on a "bulk polymer" structure. This was polyphasic, involving collagen, elastin and probably smooth muscle, in a continuous phase of polysaccharide-protein complexes. More recently (Milch, 1966), he compared the solvent action of several carbohydrate polymers on goatskin collagen with respect to ultimate tensile strength and apparent elastic modulus. An unoxidized carbohydrate polymer (dextran-sulphate) at pH 7.2 produced a three-fold decrease in tensile strength and elastic modulus, in comparison with water and oxidized polymers (e.g. dialdehyde starch). On this hypothesis removal of mucopolysaccharide enzymatically need have no weakening effect on the native tissue. It is of interest that those conditions associated with increased fragility of aorta (copper

deficiency, lathyrism and Marfan's syndrome) also exhibit increased amounts of mucopolysaccharide.

The increased aortic mucopolysaccharide of copper deficiency is probably not a primary abnormality but rather a development "pari passu" with a defect in elastin. Its place in maintaining the integrity of the medial framework of the aorta and the significance of its increase in copper deficiency, remain obscure.

Experiment 6.

The repair of vascular defects in copper-deficient swine treated with copper

Although the prevention of aortic defects by control diets of the same basic composition containing copper has been explicitly demonstrated in the foregoing experiments, the reversal of the defects by copper after the deficiency was established remained to be documented. A prompt remission of severe anaemia within three weeks of refeeding copper to deficient swine has been reported by Lahey et al. (1952). The fibrous repair of intimal fissures of the aorta in young copper-deficient swine treated with copper has already been alluded to. The final study was undertaken to determine the extent of vascular repair and restoration of mechanical properties in vessels after reversal of copper deficiency in young swine. I am indebted to Dr. W. H. Carnes for the electron microscopic preparation.

Experimental

Several litters of mixed breed swine, aged 2 to 4 days, were received at different times from the same breeder. They were divided into a copper-deficient group of 34 and a control group of 3 pigs. All the animals received supplementary iron and other minerals. They were weighed and bled for measurement of volume of packed red cells at weekly intervals. Nineteen of the copper-deficient pigs were left untreated and, of these, twelve died at from 46 to 94 days and seven were killed at from 50 to 84 days. The remaining 15 copper-deficient animals were left until the

degree of anaemia and physical signs indicated impending death, whereupon copper was added to their diet in the same dosage provided the control pigs. The treated animals were killed at intervals of 10 days, 2 weeks, 4 weeks, 5 weeks and 6 weeks after beginning copper supplements. Their ages ranged from 59 to 131 days at time of death. The control animals were killed at ages of 46 to 110 days. Aortic tensile strengths were measured routinely. Segments from the coronary, splenic, hepatic, anterior mesenteric, pancreatic, renal, carotid, femoral, brachial, thoracic and abdominal aortic and pulmonary arteries were fixed in Helly's fluid and, after paraffin embedding, were sectioned and stained with haematoxylin and eosin, and by the Verhoeff-Van Gieson method for elastin.

Selected samples for electron microscopy, taken from right and left main coronary arteries, were fixed in cold 3% glutaraldehyde in Millonig's phosphate buffer at pH 7.4 overnight, washed in the cold buffer and trimmed to blocks less than 1 mm in greatest dimension. The blocks were stored in the buffer variable periods of time and then postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded ethanol concentrations, cleared with propylene oxide and embedded in Epon 312. Ultra-thin sections were stained with saturated uranyl acetate in 50% ethanol and examined with the Bendix Tronscope-TR50.

Results

The volume of packed red cells in the control pigs was 40 to 45%, whilst it fell progressively in the deficient pigs to below 20% at death. At the time of reversal of the deficiency this

value ranged between 10% and 21% in the copper treated group. Following treatment there was rapid rise to normal levels by 3 weeks (Fig. 52).

The tensile strength of the control aortas was 16.5 ± 2.8 kg/cm² while that of the deficient animals varied between 2.4 and 5.1 kg/cm². The aortic tensile strength of the treated group varied widely between 5.3 and 18.6 kg/cm² (Fig. 53).

Figure 54 illustrates the response of aortic tensile strength to copper therapy as a function of time. After adding copper to the diet there was progressive strengthening, especially between the second and third weeks, levelling after the fourth week in the control range.

In the animals dying with copper deficiency, lesions were encountered like those of previous experiments. Figure 55 illustrates the abdominal aorta of a deficient animal undergoing haemorrhagic dissection at the apex of a fissure. This process interrupts elastic laminae which are further fragmented and separated by smooth muscle cells, collagen and abundant amorphous matrix, some of which takes the Verhoeff haematoxylin stain for elastin. After four weeks of copper treatment, a similar aortic fissure still shows distorted and fragmented laminae, but the muscle cells are now plump and active with a mesh of delicate elastin fibres in a pericellular distribution (Fig. 56). The excess of amorphous matrix is no longer visible.

Figures 27, 30 and 31 illustrate the changes in a splenic artery from an animal dead of copper deficiency. The internal elastic lamina is interrupted at several points. Repair of such lesions appears to be effected in two ways in the copper treated

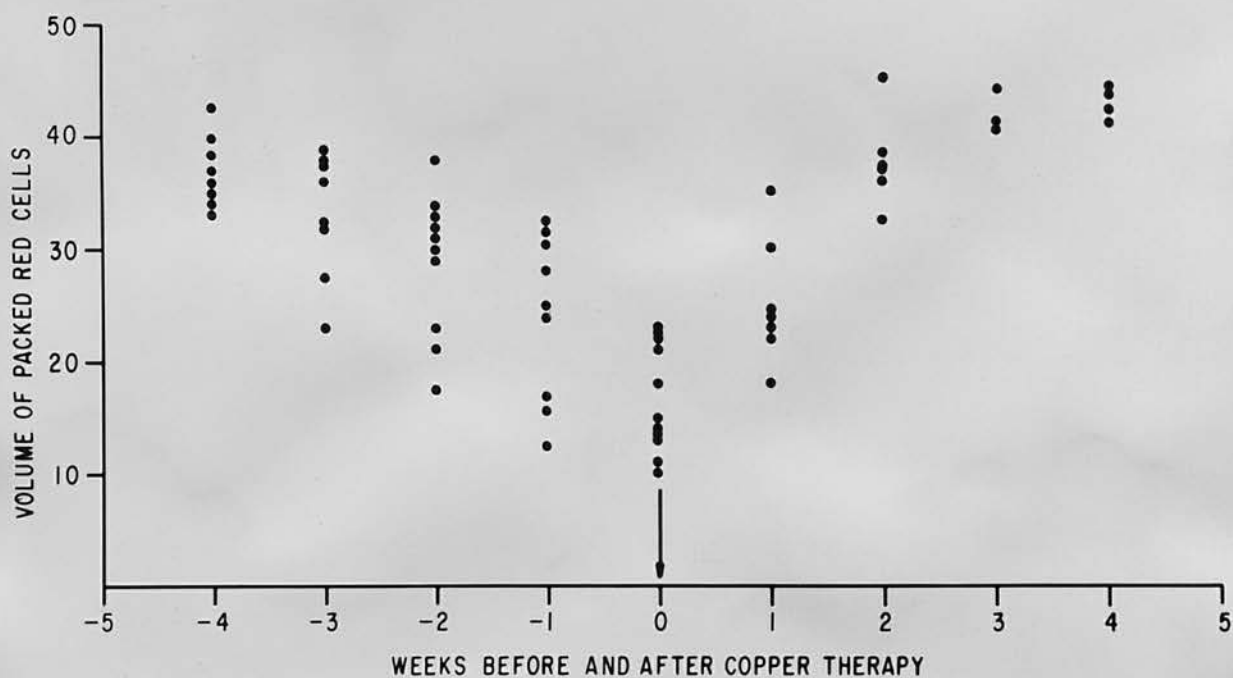


Fig. 52. Haematocrit readings at weekly intervals reflecting the development of and recovery from anaemia in copper-deficient pigs given copper supplements at time indicated by arrow.

TENSILE STRENGTH OF AORTA

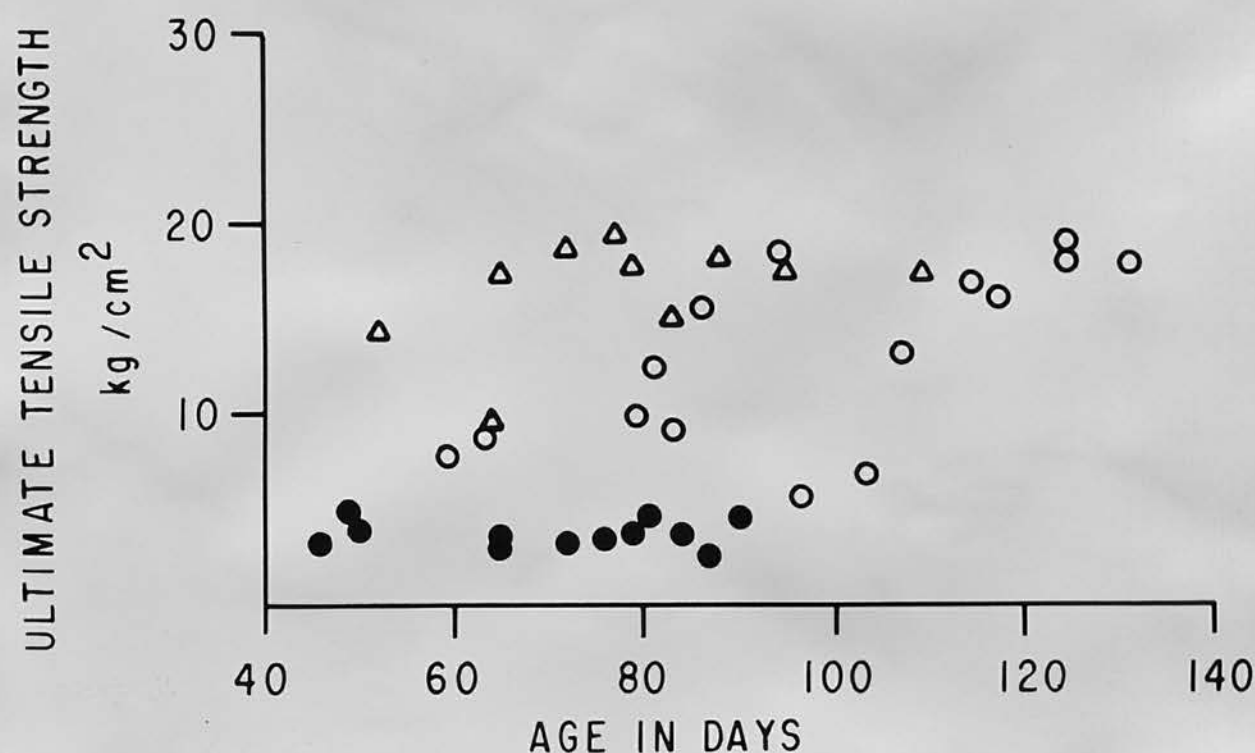


Fig. 53. Tensile strength of aorta in control pigs (Δ), in pigs sacrificed when copper-deficient (\bullet), and in pigs which had been fed copper after development of the deficiency (\circ), as a function of age.

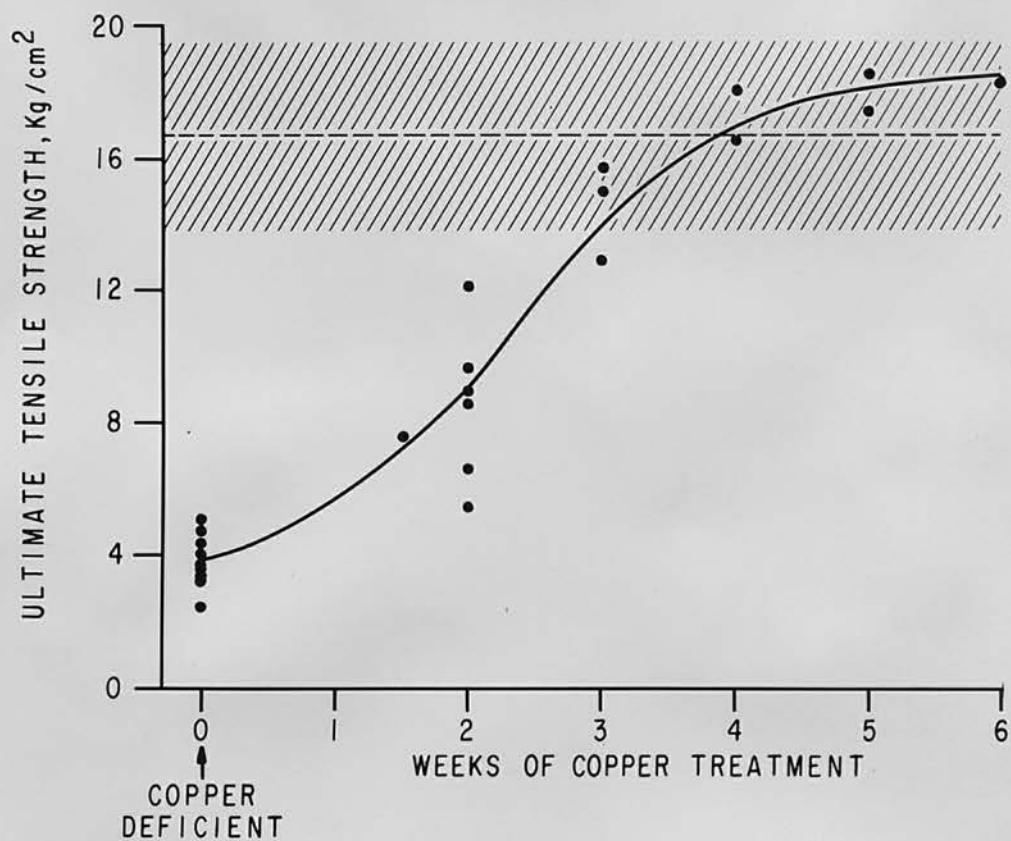


Fig. 54. Tensile strength of aorta in pigs which were fed copper after establishment of the deficiency. Dashed line and shaded area indicate mean and standard deviation of control values.

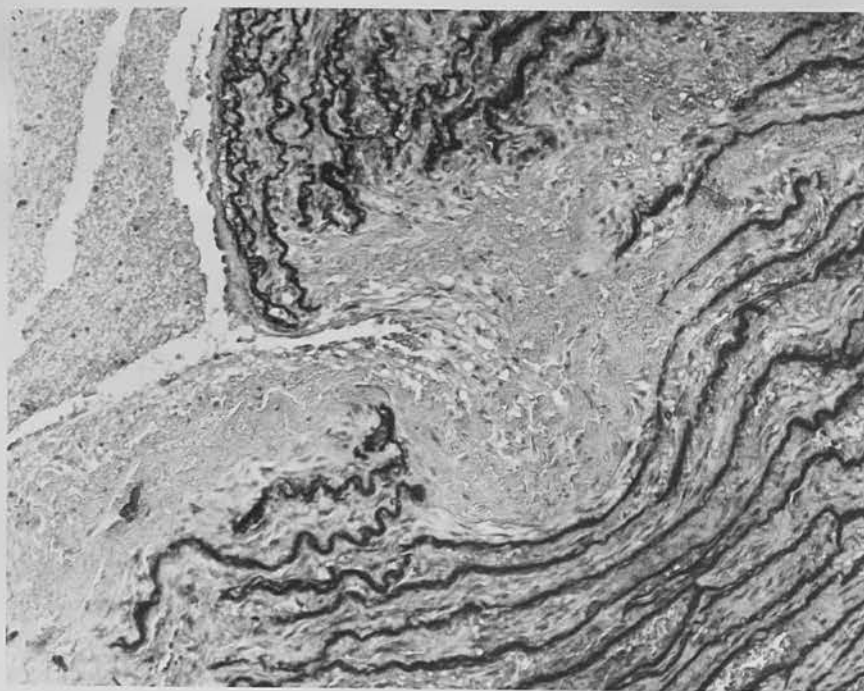


Fig. 55. Aorta of deficient pig (#12), showing recent fissure of inner layers interrupting medial elastic laminae and filled with amorphous material. Verhoeff-van Gieson stain. x 150.

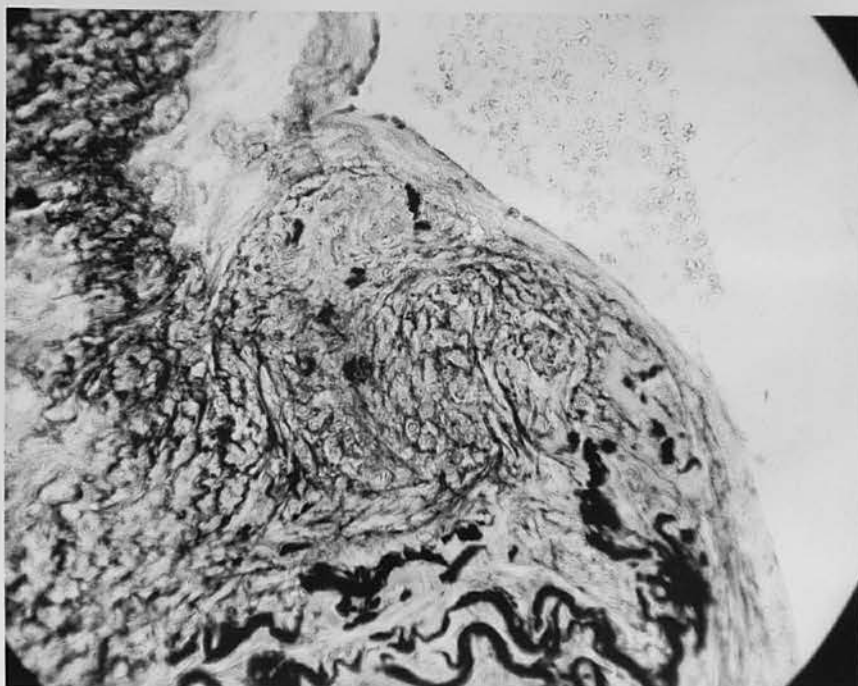


Fig. 56. Aorta of pig (#24) treated with copper for 28 days following establishment of deficiency. The fissure has been filled in by cells and intercellular fibres which stain like elastin. Verhoeff-van Gieson stain. x 250.

animal. The first is a re-establishment of continuity by end-to-end formation of new elastin. Figures 57 and 58 illustrate parts of splenic arteries from pigs fed copper for 11 days and four weeks respectively after severe deficiency had been established. The ends of the thick wavy elastica are joined by a fine, highly convoluted membrane of more recently formed elastin. In the second, there is formation of a new membrane parallel to the old. The hepatic artery in Fig. 59 is from an animal after three weeks of therapy. Internal to the original and fragmented elastica, there is a new, as yet incomplete lamina separated by radially oriented intimal cells. Duplication of the internal elastic lamina is occasionally seen in vessels from control animals, but the two membranes are always qualitatively identical and not associated with laminar breaks. In addition to the repair of the internal elastic lamina, intimal plaque formation similar to that already described in Experiment 1 became more obvious and qualitatively changed. Figure 60 illustrates a plaque overlying a large break in the internal elastic lamina of a coronary artery from a pig fed copper for 6 weeks after the establishment of deficiency. A higher power view of this lesion (Fig. 61) shows the same extensive, peri-cellular deposition of elastin as seen in the aortic lesion (Fig. 56). Figures 62 and 63, from a mesenteric artery after 3 weeks of therapy, show a dense network of elastin fibres which may be compared with their relative paucity in the coronary arterial plaque of an untreated deficient pig illustrated in Figure 32.

Electron micrographs of affected coronary arteries confirmed the nature of the new membranes staining with Verhoeff haematoxylin

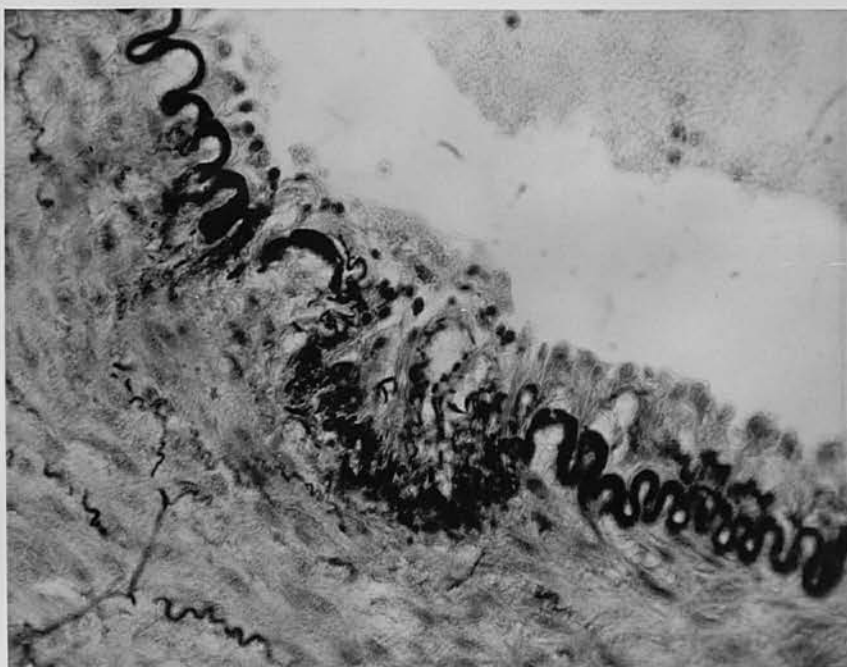


Fig. 57. Splenic artery of pig (#44) treated with copper for 11 days following establishment of deficiency. Interruption in original thick internal elastic lamina has been filled in by finer fibres or membranes staining like elastin. Verhoeff-van Gieson stain. x 250.



Fig. 58. Splenic artery of pig (#1626) showing elastin-staining thin fibres or membranes joining broken edges of original internal elastic membrane. Verhoeff-van Gieson stain. x 500.

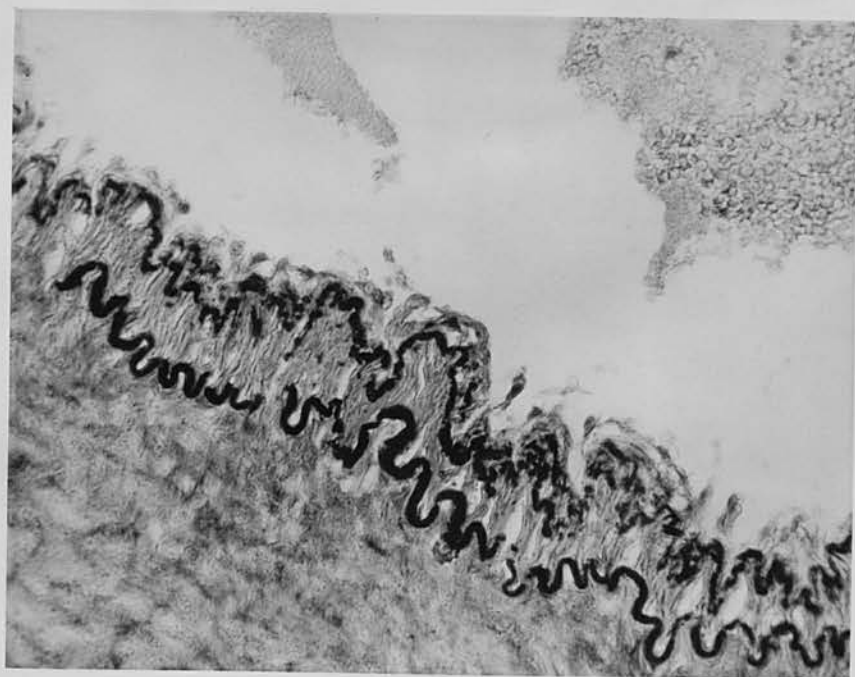


Fig. 59. Hepatic artery of pig (#23) treated with copper for 21 days following establishment of the deficiency, showing duplication of internal elastic membrane. The new membrane (above) is thinner and less regular than the old. Verhoeff-van Gieson stain. x 250.



Fig. 60. Coronary artery of pig (No. 29) treated with copper for 6 weeks following establishment of deficiency. A large intimal plaque bulges into the lumen. A broad hiatus in the internal elastic membrane lies beneath the plaque. Verhoeff-van Gieson stain. x 100.

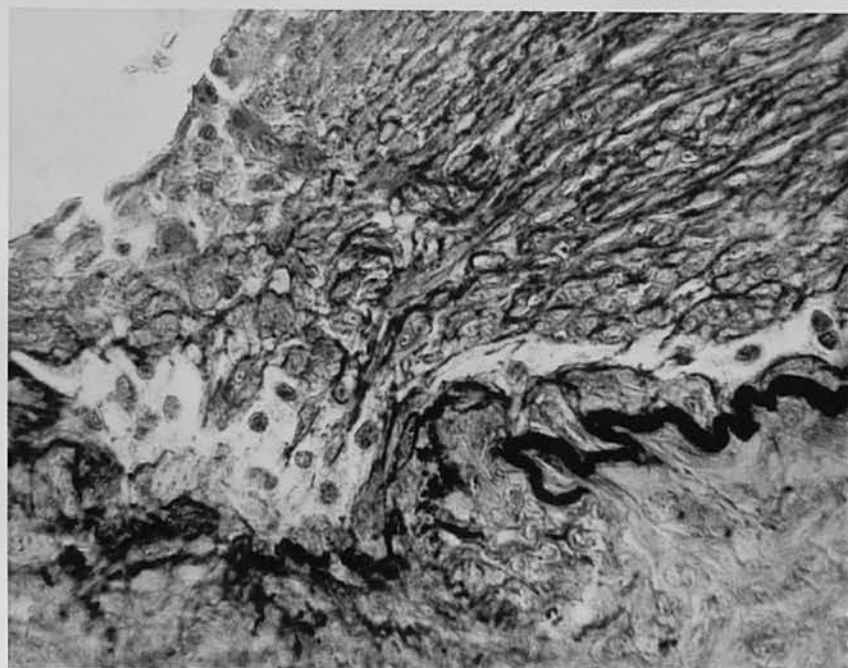


Fig. 61. Detail of lesion in Fig. 60, showing the edge of the broken original internal elastic lamina and the plump cells and intervening elastin-like material of the intimal plaque. Verhoeff-van Gieson stain. x 500.

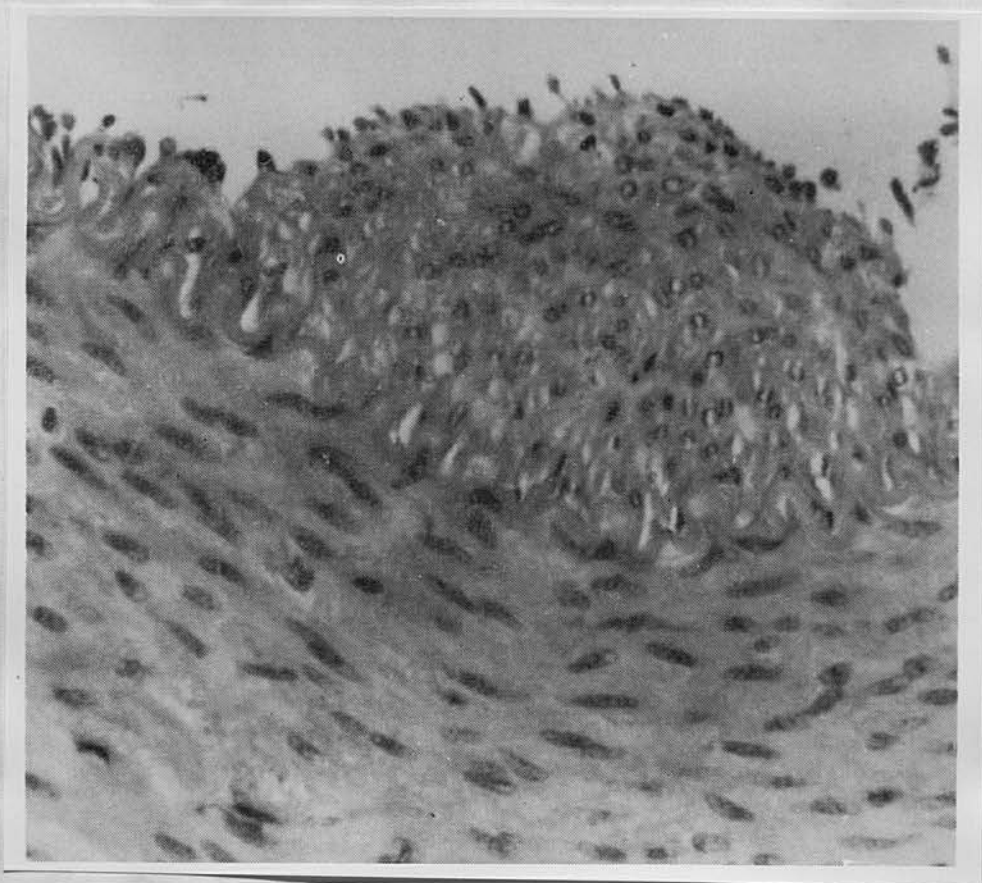


Fig. 62. Mesenteric artery of pig (No.11) treated with copper for three weeks after establishment of deficiency. A mound-like cellular thickening of the intima is covered by endothelium. Haematoxylin and eosin; x 400.

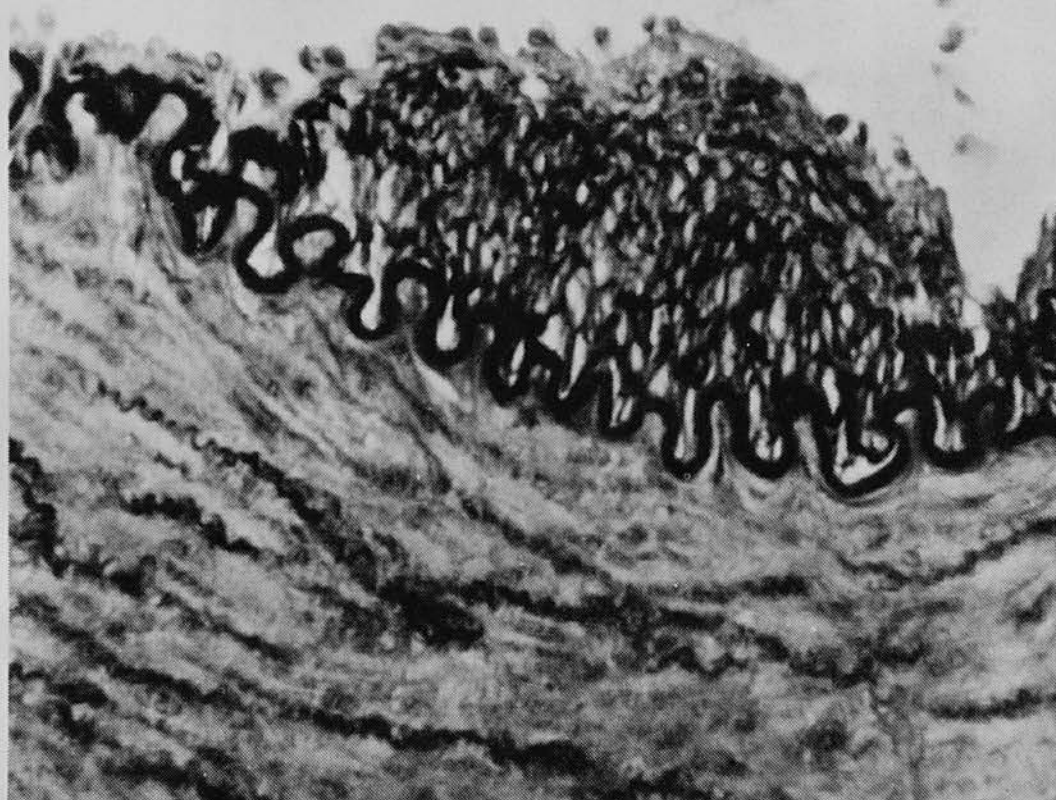


Fig. 63. The same intimal thickening shown in Fig. 62 contains a network of elastin fibrils. The break in the internal elastic membrane is not shown at this level. Verhoeff-van Gieson stain; x 400.

in the copper treated pigs. Figure 64 shows a convoluted segment of thin membrane, similar to that in Figs. 57 and 58, joining the edges of a broken internal elastic membrane (outside the field of the picture). The irregular, nonfibrillar band of low density is surmounted on its intimal surface by a densely stained layer of unresolved structure. The excess of sub-endothelial amorphous reticulated substance and the space beneath the membrane contain sparse, densely stained collagen fibrils.

Discussion

The chief importance of this experiment was the demonstration of the reversibility of the copper-deficient state and of the restoration of mechanical strength of the aorta. The rapid regeneration of elastic membranes reinforced the earlier evidence that the defect in elastin in copper deficiency was indeed specifically related to copper. It suggested further that the defect involved the biogenesis of elastin. Recent advances in the knowledge of the structure of elastin now offer a plausible hypothesis to explain this defect as a consequence of the failure of certain oxidative steps in the biosynthesis of elastin.

The insolubility of elastin in all reagents except those that break peptide bonds, and its rubber-like mechanical properties led to the conclusion that it could be regarded as a cross-linked polymer gel, containing long peptide chains, randomly crumpled, and held together laterally at intervals by strong chemical bonds (Partridge, 1962). The nature of these cross-linkages was elucidated by the isolation, from among the products

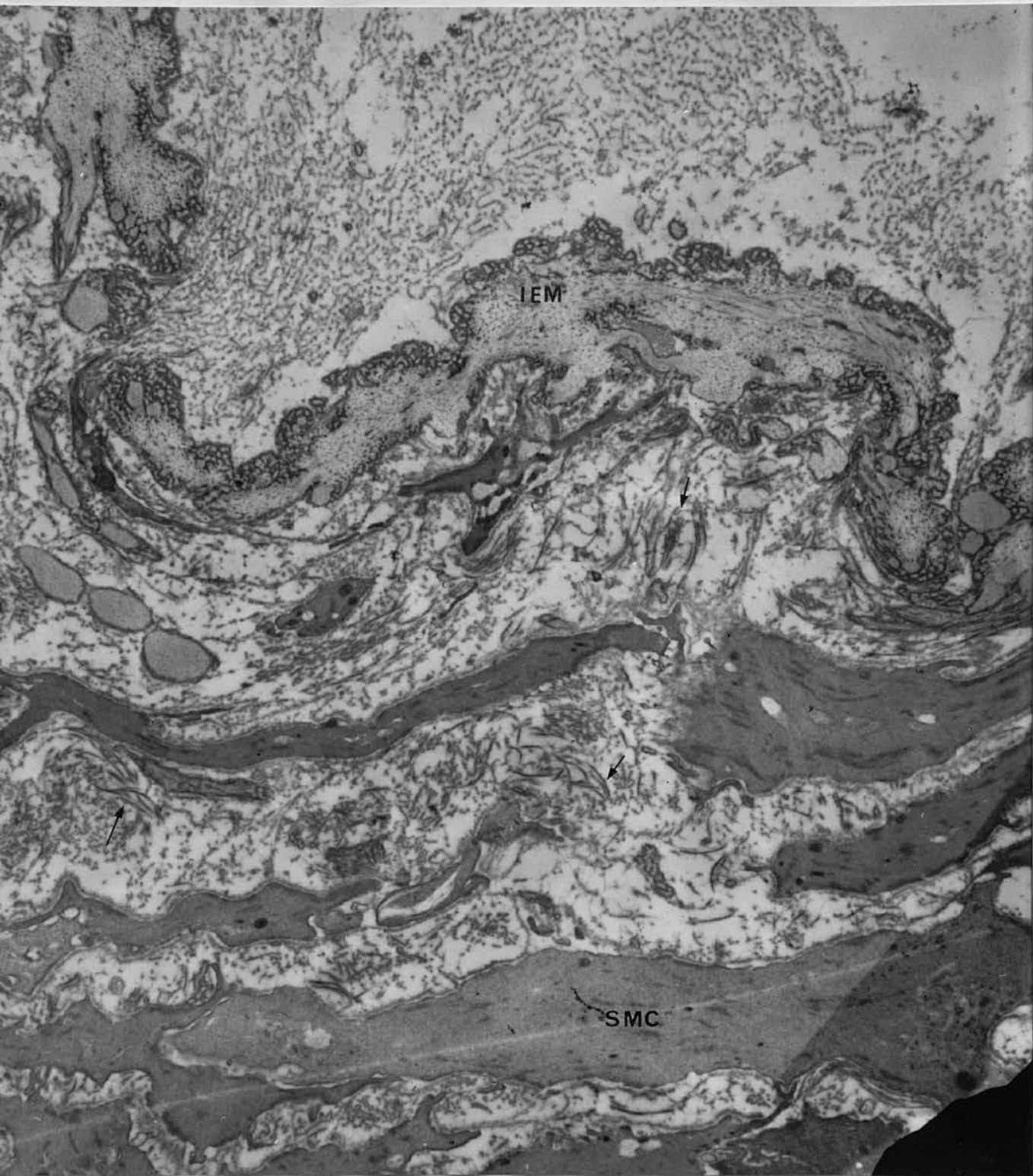
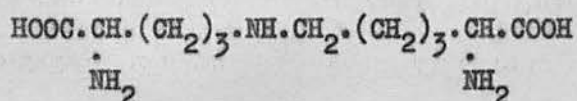


Fig. 64. Electronmicrograph of coronary artery of pig (444) treated with copper for 11 days following establishment of the deficiency. The reforming internal elastic membrane (IEM), collagen fibres (arrows) and smooth muscle cells of the media (SMC) are shown. Uranyl acetate stain. x 10,000.

of enzymic hydrolysis of elastin, of two heterocyclic amino-acids, which retained its characteristic chromophoric properties (Partridge et al., 1963; Thomas et al., 1963). The two compounds were shown to be isomeric N- and C- substituted pyridines, carrying four saturated aliphatic chains with α -amino-acid terminals. They were designated desmosine and iso-desmosine. Their structures suggested that either molecule could be formed from four lysine residues as the result of a condensation reaction. This involved the loss of the ϵ -amino group of three lysines, whilst the fourth provided its ϵ -amino nitrogen to complete the pyridinium ring. Confirmation of the origin of the desmosines from lysine was provided independently by Partridge et al. (1964) and by Miller et al. (1964), both using lysine- C^{14} . The structures of desmosine and iso-desmosine are given in Fig. 65. Subsequently, a further cross-linking amino-acid was postulated by Franzblau et al. (1965), with the structure: N^ϵ -(5-amino 5-carboxypentanyl)-lysine (lysinonorleucine).



This proposition received confirmation from Cleary et al. (1966) who reported the occurrence in elastin of two other compounds, as yet unidentified, which also incorporated radioactive lysine.

Miller et al. (1964) showed that the amino acid composition of chick aortic elastin did not change with age except for a progressive decrease in lysine and an equivalent increase in desmosine and iso-desmosine content. This suggested that peptide-bound lysine was gradually cross-linked and implied the existence

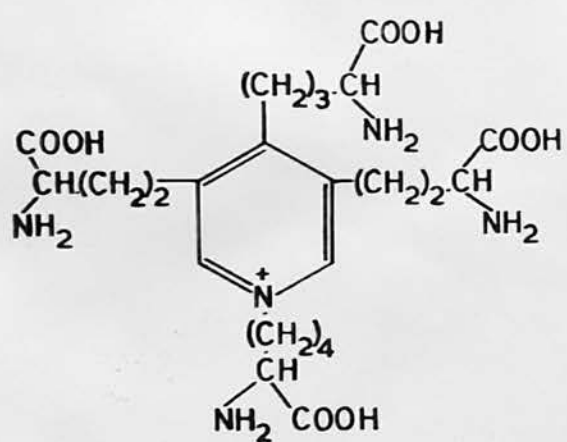
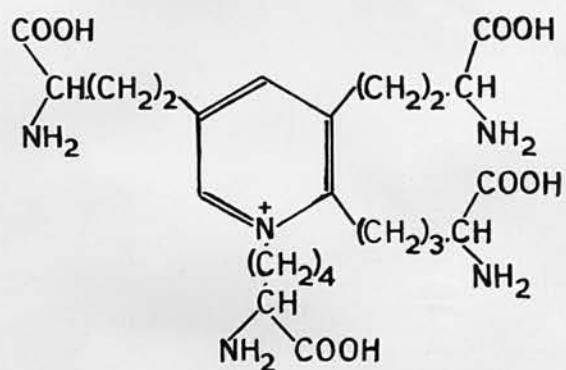
DESMOSINEISODESMOSINE

Fig. 65

of a sparsely cross-linked precursor of insoluble elastin. Such an interpretation was supported by the progressive rise in the ratio of specific activity of desmosines to that of lysine in the aortic elastin of embryonic chick aorta, pulse labelled with lysine- C^{14} (Miller et al., 1964).

The same group (Miller et al., 1965) working with copper-deficient chicks demonstrated a lowered content of the desmosines in aortic elastin, and at the same time an increased lysine content, confirming the earlier report of Starcher et al. (1964). A similar conclusion was reached by O'Dell et al. (1966). This finding suggested a block in the oxidative deamination of lysine, a reaction that is postulated to be preliminary to the formation of desmosine cross-linkages. A retardation in the conversion of lysine to desmosines in the aortas of copper-deficient swine has not yet been reported. The increased lysine content (Weissman et al., 1965) and the reduced desmosines (Weissman, personal communication) of aortic elastin from copper-deficient pigs is good evidence that this is the mechanism for the cross-linking of elastin in the pig. The presence of an insufficiently cross-linked elastin in copper deficiency would account for the increased solubility in formic acid and for the lowered tensile strength, which are related to the degree of covalent cross-linking (Wood, 1954). It might also explain the nature of the buffer soluble component of aortic elastin from copper-deficient pigs reported by Weissman et al. (1963) although recognition of this as a "soluble elastin" analogous to soluble collagen awaits detailed amino acid analysis.

If the failure of condensation of peptide-bound lysine to

desmosine cross-links is the central defect in elastin metabolism in copper deficiency, the role of copper still needs clarification. The oxidative de-amination of the ϵ amino group of lysine is postulated to be the first step in the reaction. This would yield a semi-aldehyde which, when stabilised in a suitable steric configuration, could condense to the ring formation (Partridge, 1965). A reduction in detectable free aldehyde groups of aortic elastin in copper-deficient chicks (Miller and Fullmer, 1966) strengthens this hypothesis. The diamine oxidases are copper enzymes (Yamada and Yasunobu, 1962). A similar enzyme of pea seedlings (Mann, 1961) has been shown to catalyze the formation of heterocyclic compounds, including pyridines, from aliphatic amines (Mann and Smithies, 1955). Its activity toward lysine, however, has not been reported, and Buffoni and Blaschko (1964) do not mention any activity, in this respect, of pig plasma diamine oxidase. However, it may be significant that the plasma diamine oxidase of pigs is markedly lowered in copper deficiency and is restored promptly upon treatment of the deficient pigs with copper (Blaschko et al., 1965). This observation has been confirmed in chicks by Kim and Hill (1966), who also demonstrated the greater incorporation of radioactive lysine into desmosines, in organ culture of the aorta, in the presence of hog kidney diamine oxidase.

It is of great interest that similar biosynthetic pathways are now considered to be utilised in the cross-linking of collagen. Bornstein et al. (1966) have demonstrated the presence of a lysine-derived aldehyde in peptides derived from rat skin collagen. They postulate an aldol type condensation of two aldehydes to form

an intramolecular cross-link of α -chains. Blumenfeld and Gallop (1966) claim the existence in fish collagen of another cross-linking compound, di-enosaline, which also requires the oxidative de-amination of peptide-bound lysine for its formation. The various lathyrogens such as β -aminopropionitrile (BAPN) have been shown to inhibit the intramolecular cross-linking of collagen (Martin et al., 1963). Evidence has been presented that BAPN prevents the oxidative de-amination of peptide-bound lysine to the intermediate aldehyde (Bornstein et al., 1966). Lathyrogens also inhibit the formation of desmosines in elastin (Miller et al., 1965; O'Dell et al., 1966). Now the claim has been made by Page and Benditt (1966) that BAPN in concentrations which produce lathyrism in the chick embryo, competitively inhibits an amine oxidase of the type which may be involved in collagen and elastin cross-linking. It seems therefore that a copper enzyme may be involved in the metabolism of both elastin and collagen. If copper deficiency does reduce the cross-linking of collagen, no experiment so far has demonstrated it. Moreover, although similarities exist between copper deficiency and lathyrism, especially in the aortic lesions, the two diseases are in many ways quite different. The individual steps in the conversion of the ϵ -amino group of peptide-bound lysine to a covalent cross-link at either intra- or inter-molecular level, probably involves more than one enzyme. The evidence strongly favours the association of copper deficiency with inhibition of oxidative de-amination of lysine at the ϵ position. It is still possible that BAPN may inhibit an enzyme elsewhere in the reactive pathway, producing a similar though not identical disease. Both copper

deficiency and lathyrism resemble Marfan's syndrome in their aortic lesions. Although there is no question of lathyrotic intoxication or copper deficiency in this syndrome, it is also possible that the disease is caused by the genetic absence of one of the enzymes involved in the cross-linking mechanism of elastin or collagen.

After trying to assess the parts played by the various components of the aortic wall in copper deficiency, one still remains, probably the most important of all - the living cells. In the media these are spindle-shaped and electron microscopically they have been identified as smooth muscle cells (Ham, 1962; Paule, 1963; Seifert, 1963). The cell population is uniform.

Their role in the mechanical properties of the aorta is probably less important than that of elastin and collagen, particularly with respect to tensile strength in the relaxed state. It is the opinion of most, that in this state the muscle cells behave as a viscous rather than an elastic element (Zatzman et al., 1954; Milch, 1965; Wiederhielm, 1965). This applied to the stretching experiments described in this study. During active muscular contraction, however, elastic moduli, if not tensile strengths, may be modified (Peterson et al., 1960; Apter, 1966).

Of much greater importance is the realisation that these cells must be the source of the matrix fibres and ground substance. This assumption had in fact been made previously by Haust et al. (1960), studying the role of smooth muscle cells in the fibrogenesis of arteriosclerosis, and been reinforced by others (Biava and Bencosme, 1962; Murray et al., 1966). They are the only cells present in the aortic media. Whether or not the

fibrous proteins are completed intra-cellularly or extra-cellularly, the building "units", both structural and enzymatic, do have a cellular origin. It is tempting, therefore, to regard the vascular lesion of copper deficiency as a lesion of the smooth muscle cells of the vessel wall, in which they are deprived of the copper necessary to build an enzyme, diamine oxidase, which is required for the oxidative de-amination of peptide-bound lysine in an elastin precursor. This in turn retards the formation of covalent cross-links within the elastin precursor, which then persists as an inadequately cross-linked, defective elastin, unable to convey sufficient strength to vessels for them to withstand the pressure of blood flow.

Summary

The occurrence of sudden death in copper-deficient swine under investigation for anaemia led to a systematic study of their cardiovascular systems. Pigs were reared from birth on a copper-deficient skimmed milk diet, supplemented with iron, other trace minerals and vitamins. Seventy per cent of them died at ages ranging from 60 to 130 days with haemopericardium due to rupture of the heart, aorta, coronary or pulmonary arteries. One or more of a series of distinctive lesions, consisting of aortic intimal splits and fissures, ruptured papillary muscles, dissecting coronary haemorrhages and histological defects of the vascular elastic tissue with interlamellar deposits of material staining variably like acid mucopolysaccharide or elastin, were found in almost all the deficient animals.

Measurement of the load-extension behaviour of transverse loops of aortas from copper-deficient pigs showed a 4-fold reduction of ultimate tensile strength as compared with those of the controls. Analysis of stretch moduli suggested alterations in both the elastin and collagen components of the aortic wall. The isolation of aortic elastin by formic acid extraction revealed that the percentage of insoluble elastin residue in the copper-deficient animals was reduced to half that of the control animals. The tensile strength of the deficient elastin was about one-third that of the control. The combination of morphological and mechanical evidence together with biochemical studies, on which the author collaborated, clearly pointed to an association of copper deficiency and a defect in aortic elastin.

The mechanical properties of aortic collagen were measured after

removal of elastin by pancreatic elastase. There were considerable reductions in both ultimate tensile strengths and stretch moduli in excess of the expected losses due to the removal of elastin. The copper-deficient and control aortas behaved similarly. The conclusion was that the strength of the aorta, largely a function of the collagen component, depended also on the integrity of its elastin component. An ancillary experiment showed that skin, which derives its strength almost entirely from collagen, was not weakened in the copper-deficient pig. The enzymatic removal of the acid mucopolysaccharide ground substance produced no effect on the tensile strengths of aortas from either copper-deficient or control animals.

The reversal of copper deficiency was investigated at intervals from two to six weeks after giving oral copper to severely deficient animals. There was prompt recovery from the anaemia and from other signs of the deficiency including tensile strength of the aorta. Coincidentally with the restoration of mechanical properties of the aorta during recovery there was repair of elastic membranes by new formation of elastin.

A cellular and molecular hypothesis was given to explain the association of copper deficiency with a defect in vascular elastin. The strength and inertness of elastin may depend on covalent intra- or inter-molecular cross-linking, involving the oxidative deamination of peptide-bound lysine. This is probably achieved by a copper enzyme, diamine oxidase, the formation of which is inhibited by a state of copper deficiency. The enzyme, as well as the polypeptide precursors of elastin, is probably produced by the smooth muscle cells of the vascular tunica media.

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Having arrived in Utah in the autumn of 1960, I immediately fell heir to an opportunity to investigate the cardiovascular lesions in copper-deficient swine. This was made possible because Dr. George S. Shields, whose idea it originally was, left to take another position.

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